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# THE EFFECT OF LOCAL TESTOSTERONE ADMINISTRATION ON THE PROSTATE OF THE RAT[1]

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ABSTRACT

Pellets of crystalline testosterone or testosterone mixed with cholesterol in ratios of 1:2 or 1:5 were implanted in one lobe of the ventral prostate of castrated Sprague-Dawley rats. The weights and histological appearance of these lobes when compared to those of the contralateral prostatic lobes provide evidence for a greater stimulation or maintenance of growth of the lobes containing the androgen pellets. Inert pellets such as cholesterol had no such effect. This demonstration of a correlation between the presence of testosterone locally and the rate of growth of a lobe of the ventral prostate provides evidence for a local action of testosterone on the prostate. The technique employed in this study should be useful in a search for, and an evaluation of, substances directly inhibiting the action of androgen on the prostate.

THIS study was undertaken to evaluate the effect of the local application of an androgen on the prostate. The growth promoting effect of the local application of sex hormones to secondary sex organs is well known The response of the comb of the chick (1) and the capon (2) to topically applied androgens has been repeatedly demonstrated. Lacassagne *et al.* (3) and Robson (4) reported histological evidence of stimulation of the epithelium of the seminal vesicle of the rat after depositing testosterone propionate in a vesicle. Similarly, the local application of estrogen has been demonstrated to result in stimulation of the vagina, uterus, and mammary gland of several species (5). However, the prostate has not been shown to respond locally to an androgen administered within the gland.

The ventral prostate of the rat seemed to offer an excellent site for an attempt to evaluate the effect of local androgen administration on the prostate. The ventral prostate consists of two symmetrical lobes each with a separate blood supply and duct system. It seemed likely that the implanta-

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tion of an androgen in one lobe of the ventral prostate in a castrate ra would allow a differentiation of the effect of the locally active hormon from the effect of the androgen entering the general circulation. The size o the lobe containing the androgen would be expected to represent a summation of the effects of the circulating and local androgen; whereas, the siz of the contralateral lobe would be expected to represent the effect of th circulating androgen only. The following experiment was designed to tes this hypothesis.

## MATERIALS AND METHODS

In this experiment, 71 male Sprague-Dawley rats weighing 235 to 290 grams were used. At the onset of the experiment, 8 rats (Group 1) were sacrificed with ether and autopsied. The body weight and the weights of each lobe of the ventral prostate and the posterior prostate were determined. All the remaining rats were anesthetized with ether and the prostate was exposed through a lower midline abdominal incision. In 27 animals, simple manipulation of either the left or the right lobe of the ventral prostate was carried out; in the other 36, pellets were implanted in either the right or the left lobe of the ventral prostate. Within each group of rats, the lobe of the ventral prostate that was manipulated or in which a pellet was implanted was alternated in successive animals. At the time the prostate was exposed, bilateral orchiectomy was performed in all rats except 8. These 8 rats (Group 2) and 6 rats (Group 3) subjected to castration as well as manipulation of the prostate served as intact and castrate controls respectively. The remaining 13 rats subjected to simple manipulation of the ventral prostate had pellets implanted subcutaneously. In 7 of these rats (Group 4), a pellet of compressed crystalline testosterone weighing 2.1 to 4.1 mg. was placed subcutaneously; in 6 (Group 8), a 2.4 to 4.5 mg. pellet consisting of 1 part crystalline testosterone and 5 parts cholesterol was implanted subcutaneously.

Of the 36 rats with a pellet implanted in one lobe of the ventral prostate, 6 (Group 9) had a pellet of cholesterol weighing 2.2 to 4.0 mg. implanted. In 10 rats (Group 5), a crystalline testosterone pellet weighing 2.4 to 4.1 mg. was implanted in the ventral prostate. In 10 rats (Group 6), a pellet consisting of 1 part crystalline testosterone and 2 parts cholesterol and weighing 1.9 to 4.5 mg. was implanted in the ventral prostate. In 10 rats (Group 7), a pellet consisting of 1 part testosterone and 5 parts cholesterol and weighing 2.5 to 4.8 mg. was implanted in the ventral prostate.

Twenty-one days after the implantation of pellets, the animals were sacrificed with ether. During the experiment, all groups of rats showed approximately the same rate of body weight gain. At autopsy, the body weight and the weights of the individual lobes of the ventral prostate and the posterior prostate were determined. After weighing, all prostatic lobes were fixed in formalin. The pellets which had been removed prior to the weighing of the prostatic lobes were dried and weighed. However, adherent tissue prevented the final pellet weights from being quantitative. Representative prostatic lobes were selected for sectioning and histological examination in each group.

A second experiment was performed in an effort to evaluate the influence of an inert pellet such as cholesterol on the prostate in the presence of androgen stimulation. In this experiment, a pellet of cholesterol was placed in one lobe of the ventral prostate in 14 (Group 11) of 30 male Sprague-Dawley rats. In the remaining 16 rats (Group 10) either the right or the left lobe of the ventral prostate was exposed and manipulated. At the time the prostate was exposed, a bilateral orchiectomy was performed and testosterone pellets were implanted subcutaneously in all rats. The experimental procedures to which these 30 rats were subjected resembled those of the initial 71 rats in all other details.

## RESULTS

The results are shown in Table 1. Inspection of these results shows that there is a striking similarity in the weight of each of the two lobes of the ventral prostate in those instances in which no pellet was implanted in one of the lobes. This is evident in the rats autopsied initially (Group 1) as well as those autopsied after manipulation (Group 2). It is as apparent in rats subjected to endogenous androgen only (Group 2) as it is in rats with subcutaneously implanted testosterone (Group 4) or cholesterol-testosterone pellets (Group 8). In contrast to this, the disparity in weight between the

TABLE 1. RESULTS OF IMPLANTATION OF TESTOSTERONE IN VENTRAL PROSTATE OF RATS

| | Group | Number of rats | Final body weight (gm.) | Manipulated[1] lobe of ventral prostate (mg.) | Non-manipulated lobe of ventral prostate (mg.) | Difference manipulated and non-manipulated lobe of ventral prostate (mg.) | % Manipulated/non-manipulated ventral lobe of prostate | Posterior prostate (mg.) |
|---|---|---|---|---|---|---|---|---|
| | Intact controls | | | | | | | |
| 1 | Autopsy, Day 0 | 8 | 266 | 204 | 195 | +9[2]± 9* | 105% ± 5 | 186±12 |
| 2 | Autopsy, Day 21 | 8 | 346 | 260 | 269 | −10±11 | 97% ± 4 | 256±11 |
| | Castrate | | | | | | | |
| 3 | ——— | 6 | 331 | 30 | 28 | + 2± 2 | 108% ± 6 | 58± 7 |
| 4 | Testosterone, Subcutaneous | 7 | 321 | 299 | 301 | − 2±15 | 99% ± 4 | 325±22 |
| 5 | Testosterone in Prostate | 10 | 312 | 410 | 353 | +56±13 | 118% ± 5 | 349±20 |
| 6 | Testosterone:Cholesterol (1:2) in Prostate | 10 | 329 | 214 | 137 | +77± 9 | 160% ± 9 | 146± 8 |
| 7 | Testosterone:Cholesterol (1:5) in Prostate | 10 | 321 | 143 | 66 | +77± 9 | 300% ±77 | 70± 6 |
| 8 | Testosterone:Cholesterol (1:5), Subcutaneous | 6 | 318 | 87 | 87 | 0± 2 | 99% ± 2 | 78± 5 |
| 9 | Cholesterol in Prostate | 6 | 304 | 25 | 21 | + 4± 1 | 116% ± 4 | 39± 2 |
| *Experiment 2* | | | | | | | | |
| 10 | Testosterone, Subcutaneous | 16 | 329 | 341 | 351 | −11+12 | 98% ± 4 | 403±11 |
| 11 | Testosterone, Subcutaneous; Cholesterol in Prostate | 14 | 322 | 301 | 313 | −13±15 | 98% ± 5 | 379±18 |

[1] "Manipulated" used to designate the lobe of the ventral prostate which has had pellet implanted or has been sham operated.
[2] Represents the average of individual differences.
* Standard Error.

lobe of the ventral prostate containing the testosterone or testosterone-cholesterol pellets and the opposite lobe is readily evident (Groups 5, 6, 7). There was not a single rat in which the lobe containing the androgen pellet failed to equal or exceed the lobe which did not contain the pellet. The average weight of the androgen containing lobes of the ventral prostates exceeded the average weight of the non-manipulated lobes in every group. On an absolute scale the difference was about equally evident for the groups containing crystalline testosterone or cholesteral-testosterone mixtures in the prostatic lobes. The average differences in weight between the testosterone-containing lobes and the contralateral lobes of the ventral prostates were significantly (P. .004) greater than the average differences in weight between the lobes of the ventral prostates of the rats with testosterone pellets implanted subcutaneously. On a relative scale with the weight of the lobe containing the pellet being expressed as a percentage of the opposite lobe, the maintenance of size was more evident in the groups containing pellets consisting of 1 part testosterone to 2 parts cholesterol

(160% ±9) or 1 part testosterone to 5 parts cholesterol (300% ±77). The presence of an inert pellet such as cholesterol implanted in a lobe of the ventral prostate has little effect on the prostatic weight in the castrated rat (Group 9) or in the castrated rats with testosterone pellets placed subcutaneously (Group 11). In general, the pellets containing cholesterol showed only a slight loss of weight during the experiment. The crystalline testosterone pellets were almost completely absorbed. Histological sections confirmed the apparent greater androgen stimulation of the lobe of the ventral prostate containing the androgen pellet.

## DISCUSSION

These experiments demonstrate that in the Sprague-Dawley rat there is a relationship between the presence of testosterone locally and the degree of prostatic growth or maintenance as judged by the weight and histological appearance of the ventral prostate. The results are compatible with the suggestion that the lobe of the ventral prostate containing the androgen pellet would reflect local as well as circulating androgen levels; whereas, the contralateral ventral lobe would reflect only circulating androgen levels. The presence of testosterone in varying concentrations in the implanted pellets allowed demonstration of the apparent effectiveness of local testosterone in stimulating prostatic growth (testosterone pellets), in maintaining the prostate (testosterone:cholesterol 1:2) and in retarding regression of the prostate following castration (testosterone:cholesterol 1:5). The failure of the pure cholesterol pellets to cause a variation in the weight of the lobe of the ventral prostate containing them in the presence of systemic androgen suggests that this is a property of the testosterone.

In this experiment, testosterone concentrations were not determined. However, it seems highly probable that the concentration of testosterone was higher in the lobe of the ventral prostate containing the testosterone pellet than in the opposite lobe. The demonstration in paired accessory sex glands of a differential rate of growth related to a highly probable differential in testosterone concentration lends weight to the argument for a direct local action of testosterone on the prostate. The observation of Robson (4) allows similar conclusions to be drawn for the action of androgens on the seminal vesicle. The application of these observations to a search for a local antagonist to the action of systemic androgen seems indicated (6).

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# PLASMA 17-HYDROXYCORTICOSTEROID RESPONSE TO HYPOTHALAMIC STIMULATION IN THE CONSCIOUS RHESUS MONKEY

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ABSTRACT

A technique for the study of plasma hormone concentrations during electrical stimulation of selected deep brain areas in conscious monkeys through chronically implanted electrodes is described. The experimental conditions permit repeated study of an individual animal during periods of rest and during a variety of control and experimental procedures, so that an animal can be considered to furnish his own control data. Stimulation of the hypothalamus is associated with an increase in pituitary-adrenal cortical activity, as judged by a marked elevation of plasma 17-OH-CS levels (20–25 μg. %/hr.). A closely similar rate of rise in plasma 17-OH-CS levels (20–25 μg. %/hr.) was observed in the same animals after intravenous injection of ACTH (16 mg./kg.). No significant changes in plasma 17-OH-CS levels were observed under normal conditions or during control stimulation of the putamen or anterior thalamus.

IT HAS become increasingly evident that the anterior pituitary-adrenal cortical system is extremely sensitive to central nervous system influences, as judged by the correlation between plasma 17-hydroxycorticosteroid (17-OH-CS) levels and emotional states in both man and the laboratory animal (1, 2, 3, 4 and 5). Little is yet known of the underlying neural apparatus which mediates these close relationships between behavior and pituitary function. Investigations of this problem have logically begun with the neural region physically closest to the pituitary, namely the hypothalamus, and it has been shown in a number of experiments with both stimulation and ablation techniques that ACTH secretion is related to hypothalamic function. Harris (6), Hume (7) and Porter (8) have reported that electrical stimulation of the hypothalamus is associated with evidence of ACTH release, as judged by indirect indices such as lymphocyte and eosinophile count. In view of the recognized ambiguity of such measurements as indices of ACTH secretion, the present experiments were undertaken to measure anterior pituitary-adrenal cortical activity, by means of the relatively specific index of plasma 17-OH-CS levels, during electrical stimulation of the hypothalamus in conscious monkeys. The limited objectives in this study include the evaluation of new technical approaches as well as investigation of hypothalamic effects on ACTH release,

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both aspects being regarded as necessary preliminary steps to a long-term study of the influences of distant neural structures, particularly limbic system components, on hypothalamic-hypophysial function.

## METHODS

Four male Rhesus monkeys, (*Macaca mulatta*), weighing between 3.0 and 4.2 kilograms were used in these experiments. Permanent electrodes were implanted in the hypothalamus, and, for control purposes, in the putamen in three animals, and the anterior thalamus in one animal. After allowing about seven days for postoperative recovery in the home cage, the monkeys were placed in a chair-type restraining apparatus (9), in which they remained during the period of experimental study lasting from two to five weeks. Each animal was allowed at least five days for adaptation to the chair before any experiments were begun. Experiments were subsequently performed once or twice weekly. All control and experimental sessions were of 90 minutes duration, and in each animal experiments were always started at the same time of day, usually 11 A.M., because of the diurnal rhythm of adrenal cortical activity. Blood samples for plasma 17-OH-CS determination were withdrawn immediately before and after each session.

A multilead electrode of the Delgado type, with seven tips vertically spaced about 2 mm. apart, was used in all experiments. The electrodes were implanted stereotaxically according to a procedure previously described by Delgado (10). The Horseley-Clarke coordinates employed can be estimated by reference to Figures 2 and 4. In each animal one array was implanted in the hypothalamus, with the distal electrode tips aimed at the infundibular region, while the proximal tips were in the region of the anterior thalamus. A second array included electrode tips in the putamen. Electrode leads were either left attached to the implanted plugs continuously while the animal was in the chair, or were attached one or two days prior to experiments involving blood measurements. The monkey was prevented from interfering with the electrode leads by closure or adjustment of the arm holes in the top plate of the chair apparatus (9). In stimulation experiments a differentiated square wave-type stimulus was used, as described by Lilly (11), in order to minimize injury to brain tissue. A stimulus of approximately 25 milliamperes current intensity, 0.1 millisecond pulse duration, 100 cycles per second, and 0.5 second train duration was delivered intermittently over a 90-minute period, in an alternating pattern of one train every 5 seconds for two minutes, then no stimulus for two minutes. Identical parameters of stimulation were used in both the hypothalamus and control areas.

Blood samples (5–7 ml.) were withdrawn from the saphenous vein into a heparinized syringe, centrifuged immediately, and the plasma stored in the frozen state until analyzed. Plasma 17-OH-CS concentrations were determined by the Nelson-Samuels method (12, 13). In each animal the normal diurnal variation of plasma 17-OH-CS levels was determined first. This was done with the stimulator turned on and the electrode leads attached to the animal's head plug, but the leads were not connected to the stimulator. The plasma 17-OH-CS response to a single intravenous injection of ACTH (16 mg./kg.) was also measured in each animal as a basis for reference in the interpretation of the responses associated with electrical stimulation of the brain. This ACTH dosage has been shown previously to produce a maximal rate of plasma 17-OH-CS rise for at least two hours in the monkey (14).

Upon completion of the experiments in the chair, the animals were nembutalized and perfused through the aorta, first with physiological saline, and then with 10% formalin. The brain was removed from the calvarium and fixed in 10% formalin for at least two weeks before blocks were cut and embedded in paraffin. Serial sections were cut 15 microns in thickness and stained with thionin or with Weil's stain. The electrode place-

ments were then reconstructed and microphotographs of selected sections showing the electrode tips were made.

## RESULTS

The effects of electrical stimulation of the hypothalamus on plasma 17-OH-CS levels in seven successive experiments on four monkeys are shown in Table 1. It is evident that marked elevations occurred in every experiment, with a mean rate of rise amounting to about 25 micrograms per cent per hour. It also appears that the rate of rise is independent of baseline level, at least within the baseline range encountered in these experiments.

By way of exploring further the specificity of this effect, experiments were carried out in three monkeys in which the putamen was stimulated

TABLE 1. PLASMA 17-HYDROXYCORTICOSTEROID RESPONSE TO STIMULATION OF HYPOTHALAMUS

| Monkey No. | Plasma 17-OH-CS levels (μg./100 ml.) | | |
|---|---|---|---|
| | Before stimulation | After stimulation | Rise during stimulation |
| 6 | 23 | 63 | 40 |
| | 24 | 63 | 39 |
| 7 | 25 | 57 | 32 |
| | 18 | 60 | 42 |
| 8 | 42 | 74 | 32 |
| | 15 | 59 | 44 |
| 19 | 18 | 59 | 41 |
| Mean | 24 | 62 | 38 |

for one hour and followed immediately by identical stimulation of the hypothalamus for an equal period, as shown in Figure 1. In all experiments, there was no change in plasma 17-OH-CS levels during putamen stimulation except for the slight drop of the normal diurnal variation. During the subsequent hypothalamic stimulation, however, the usual very sharp rise in plasma 17-OH-CS levels invariably occurred.

The ACTH response results are shown in Table 2, which also includes comparison of normal, control stimulation, and hypothalamic stimulation data. It is evident that the mean elevations of plasma 17-OH-CS levels following ACTH administration and hypothalamic stimulation are virtually identical, approximately 20–25 μg. %/hr. (14). It is equally evident that control stimulation in the putamen or anterior thalamus was not associated with any significant change in plasma 17-OH-CS level as compared with the normal diurnal values.

In an effort to obtain a rough indication of the relationship between the intensity of stimulating current and endocrine response, additional experiments were carried out in two monkeys with a current intensity of about

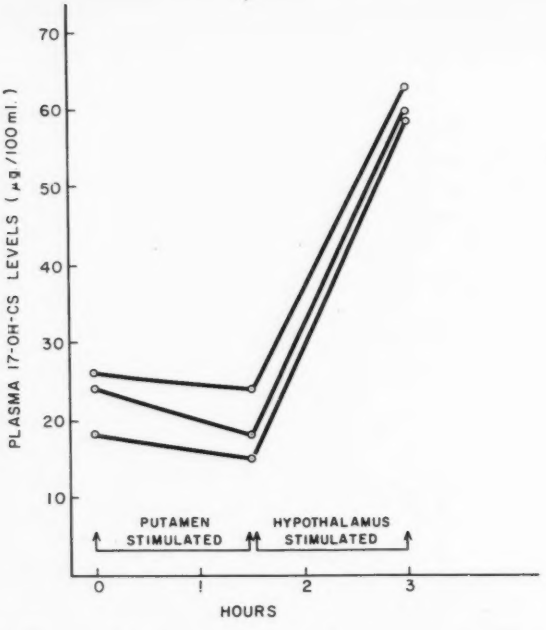


FIG. 1. Plasma 17-hydroxycorticosteroid response to hypothalamic stimulation following a control stimulation of putamen.

8 to 10 peak milliamperes. In both monkeys a lesser response was observed with the weaker stimulating current, with a mean 17-OH-CS response of 12 μg.%/hr. with 8 to 10 peak milliamperes, as compared to 25 μg.%/hr. with 25 peak milliamperes of current.

Figure 2 presents a summary of the histological studies, showing in a mid-sagittal view the estimated and the actual locations of the hypotha-

TABLE 2. SUMMARY OF MEAN PLASMA 17-OH-CS LEVELS* DURING HYPOTHALAMIC STIMULATION, ACTH INJECTION AND CONTROL PROCEDURES

| Monkey No. | Normal | | ACTH 16 mg./kg. | | Hypothalmic stimulation | | Control stimulation† | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 90 min. | 0 | 90 min. | 0 | 90 min. | 0 | 90 min. |
| 6 | 23 | 25 | 25 | 60 | 24 | 63 | 26 | 24 |
| 7 | 33 | 35 | 32 | 73 | 22 | 59 | 24 | 18 |
| 8 | 29 | 26 | 26 | 68 | 29 | 68 | 18 | 15 |
| 19 | 17 | 20 | 24 | 72 | 18 | 59 | 26 | 33 |
| Mean | 26 | 27 | 27 | 68 | 24 | 62 | 24 | 23 |
| Change during session | +1 | | +41 | | +38 | | −1 | |

* All values expressed as micrograms per 100 ml. plasma.
† Stimulation of putamen in monkeys 6, 7, and 8; stimulation of anterior thalamus in monkey 19.

lamic electrode pairs. It is apparent that three of the electrodes (M-6, M-8, M-19) were approximately 1 mm. anterior to the calculated position, but clustered fairly close together in the vertical plane. The fourth electrode (M-7), however, is displaced over 3 mm. posteriorly, lying, in fact, just behind the hypothalamus. This deviation was believed to be due to deflection of the electrode during implantation. Microphotographs of the thionin-stained cross sections showing the electrode tips in each animal

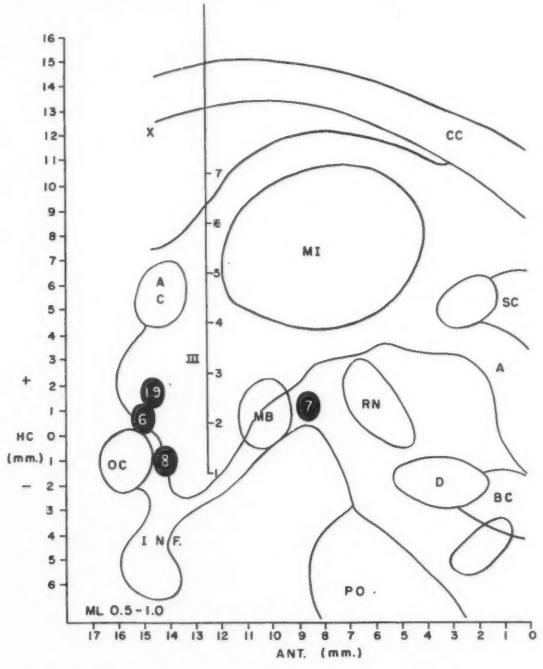


FIG. 2. Estimated and actual positions in mid-sagittal view of hypothalamic electrodes.

are presented in Figure 3 and indicate that the placements are quite close to the midline.

With regard to the electrodes used in control experiments, in three monkeys (M-6, M-7, M-8) the placements in the putamen were all within a 1 to 2 mm. radius of the estimated position shown in Figure 4. The control electrode location in M-19 lies approximately in the area marked by an "X" in Figure 2, about 8 or 9 mm. above the hypothalamic placement in this monkey. This area lies just in front of the thalamus, actually, and may have partly involved corpus callosum or even the ventricular system. The exact location of this electrode pair is made difficult by the fact that it was determined by measuring back from the hypothalamic tip of the 7-electrode array of which this was the most proximal pair.

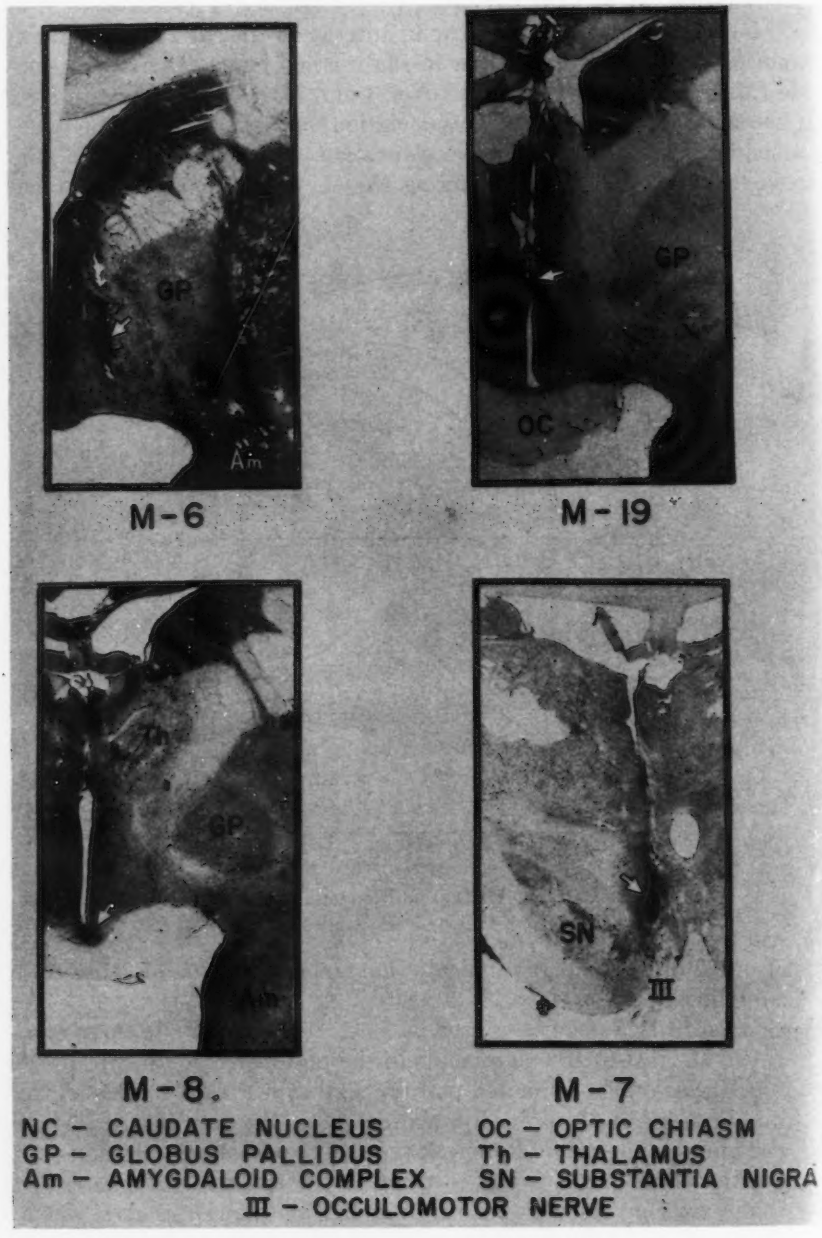


FIG. 3. Histological sections showing location of hypothalamic electrode tips.

## DISCUSSION

The primary objective in the present study was to investigate, by direct biochemical measurement of blood hormone levels, the effect of electrical stimulation of the hypothalamus on pituitary-adrenal cortical activity in the conscious animal. The consistent finding of marked plasma 17-OH-CS elevations during hypothalamic stimulation clearly supports the previous findings of several workers (6, 7, 8). Although it is admittedly hazardous to speak of "rates of rise" with only initial and terminal measurements, a reasonably strong case can be presented for considering the changes associated with hypothalamic stimulation as representing a maximal rate of rise of plasma 17-OH-CS levels. Extensive experience with a variety of conditions and agents which produce plasma 17-OH-CS elevations in monkeys has indicated with remarkable consistency that a mean rate of rise of about 20–25 μg. %/hr. appears to be maximal for this species (14, 3). This rate of plasma 17-OH-CS increase is observed for two hours after a single intravenous injection of a 4 mg./kg. dose of ACTH and no further increase in rate of rise occurs even when the dose of ACTH is increased four-fold (16 mg./kg.). Plasma 17-OH-CS levels have been measured at intervals during these first two hours and the points have invariably indicated the response to be linear during this period (14). Since this mean rate of rise of 20–25 μg. %/hr. has been repeatedly attained, but not exceeded, under the experimental conditions we have studied, it seems reasonable to look upon it again in the present case as a useful basis of reference in evaluating the significance of the observed response. Assumptions about rate of rise could not, of course, be made under conditions in which submaximal responses were observed.

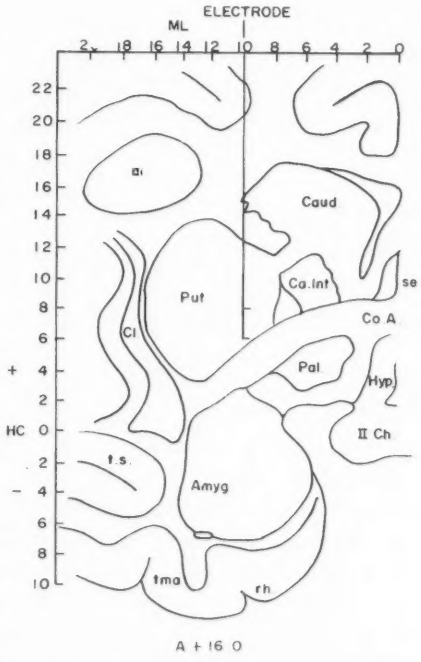


FIG. 4. Estimated positions in transverse section of electrodes in putamen.

Several points are worthy of brief comment regarding the techniques which permit experiments of this type to be carried out with unanesthetized, relatively "unstressed" animals. Considerable experience with

monkeys under experimental study in the chair-type apparatus has indicated that, after a brief, initial period of adaptation, baseline levels of activity and responsiveness of the pituitary-adrenal cortical system are very similar to those of animals kept in conventional cages (4). Day-to-day plasma 17-OH-CS levels in normal monkeys, in fact, seem to be considerably more stable, that is, vary within a smaller range, in chair-kept monkeys than in the same monkeys kept in their home cage. This may be due, in part at least, to the more rigid environmental or stimulus control which is possible in chair-kept animals, maintained in relative isolation in cubicles, in contrast to the more complex and variable environment of the central housing quarters. The present experiments support, further, that the responsiveness of the adrenal cortex to ACTH, in terms of plasma 17-OH-CS rise, in chair-kept animals is closely similar to that observed in animals kept in cages (14). It is also pertinent that the plasma 17-OH-CS response to hypothalamic stimulation of a given strength was closely reproduced in the same animal on different days often with intervening periods of two or more weeks. It appears, therefore, that considerable evidence can be presented to warrant the assumption that the experimental conditions used in this study do not in themselves appreciably alter activity or responsiveness in the pituitary-adrenal cortical system.

It is obvious that this limited study was not intended to furnish detailed "mapping" information of intra-hypothalamic localization of influences on ACTH secretion. It is of interest, however, that three of the electrodes were clustered within a relatively small area in the anterior medial hypothalamus, in the periventricular area. The location of the fourth electrode just posterior to the hypothalamus was unexpected since the plasma 17-OH-CS response in this animal was as great as the other three animals. One possible explanation of this finding could be the involvement, in this midline area, of ascending fibers from the reticular formation comprising the ascending component of the bundle of Schutz, also known as the dorsal longitudinal fasciculus. Nauta and Kuypers have recently demonstrated a considerable number of these fibers to arise in and about the dorsal segmental nucleus of Gudden and to project forward through the periaqueductal gray substance and terminate in the posterior, dorsal and infundibular regions of the hypothalamus (15). The precise relationship of these fibers to ACTH secretion remains, however, to be elucidated.

In all monkeys a rather characteristic, though somewhat subtle, behavioral response was observed during hypothalamic stimulation. Within a few seconds or minutes of the onset of stimulation the monkeys became restless and active, looking about uneasily in all directions. They appeared hyperalert, very impatient with attempts to handle them, and refused bits of favorite food, if offered to them. There was no direct observable motor response to individual stimulus bursts, however. During stimulation of the putamen no changes in overt behavior were noticed which might be attri-

luted to stimulation, except perhaps in some cases a momentary halting of ongoing motor activity during individual stimulus bursts.

On the basis of the results of this study confirming the influence of the hypothalamus on ACTH secretion, work has proceeded in our laboratory to the area of our primary interest, namely, the study of the influence on pituitary-adrenal cortical function of distant neural structures known to project to the hypothalamus, particularly some of the limbic system components. Such studies have already indicated that stimulation of the amygdaloid nucleus is associated with marked elevations in plasma 17-OH-CS levels under experimental conditions very similar to those described in the present report (16). Systematic studies currently in progress of hippocampal and reticular formation influences on ACTH secretion have yielded preliminary results indicating that these regions may also have important roles in the regulation of the pituitary-adrenal cortical system.

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# DISSOCIATION OF ADRENAL ASCORBIC ACID AND CORTICOSTERONE RESPONSES TO STRESS IN RATS WITH HYPOTHALAMIC LESIONS[1]

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ABSTRACT

A dichotomy has been observed between the adrenal ascorbic acid and corticosterone response to surgical stress in male rats with bilateral electrolytic lesions placed in the hypothalamus. Thus lesions in the posterior and mid-central portions of the hypothalamus specifically inhibited corticosterone release without altering adrenal ascorbic acid response or the weights of target organs dependent upon the normal production of pituitary hormones. Similarly, lesions in the basal tuberal region inhibited adrenal ascorbic acid depletion without affecting corticosterone release in response to stress. However, the latter lesions (which involved the median eminence) were not adrenal specific inasmuch as atrophy of the testes, seminal vesicles and prostate was also noted. Gross damage to the median eminence, basal tuberal region and portal systems blocked both adrenal responses and frequently resulted in atrophy of the thyroid and adrenal glands as well as of the sexual target organs. Water intake following hypothalamic damage was not correlated with adrenal responsiveness. Possible explanations are discussed for the ability of the adrenal to exhibit discriminatory responses to stress following hypothalamic lesions.

LESIONS in the median eminence of the hypothalamus have been reported to block or inhibit the release of ACTH as measured by adrenal ascorbic acid decline (1), eosinopenia (2) and elevated blood ACTH levels (3) in the rat, as well as corticosteroid release into the adrenal venous effluent of the dog (4, 5) and cat (6). However, a comparison of the effects of hypothalamic lesions on the corticosteroid and adrenal ascorbic acid responses to stress in the same animal has not been reported. The present studies were designed to ascertain whether lesions in the hypothalamus which blocked one of these responses uniformly inhibited the other. In addition, an attempt was made to evaluate the selectivity of lesions which inhibited adrenal responses to stress in terms of their effects on the weights of target organs of the various pituitary hormones. McCann and Brobeck (7) reported that lesions in the median eminence which damaged the supraoptic tract could be correlated with the specific block of ACTH release and

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with diuresis. In an attempt to verify this relationship, daily water intakes were also measured in all animals.

## MATERIALS AND METHODS

Adult male Sprague-Dawley rats (weighing 225–300 gm.), obtained from our own breeding colony, were used in these experiments. Lesions were made in the hypothalamus in the etherized rat placed in the Krieg-Johnson stereotaxic instrument by passing a 1–2 ma DC current[2] for 10 seconds between a 24 gauge insulated[3] nichrome wire and an indifferent rectal electrode. The bilaterally paired lesions were made at a distance less than 0.5 mm. from the midline. Anterior-posterior placement of lesions ranged from the optic chiasm to the posterior border of the mammillary body. Dorsal placement extended from 0.1–0.2 mm. from the base of the brain. Control rats were subjected to the same procedure but with no current applied. Post-operatively, rats were placed in individual cages in a temperature-controlled room. For the first 3 days ground Purina Laboratory Chow and 0.5% glucose drinking water were available *ad libitum*; whole food pellets and tap water were provided thereafter. Water intake measurements for the 24-hour period were made daily from 9:00 to 10:00 A.M. for the total 3-week post operative period. Any rat not regaining his original body weight (equal to that at the time when lesions were made in the brain) was discarded.

Three weeks after operation, laparotomy was performed on animals anesthetized with sodium pentobarbital (5 mg./100 gm. body weight) injected intraperitoneally. The preparation of the animal for adrenal vein cannulation and collection of blood was that previously described (8) with the right adrenal being removed within 1 minute after cannulation of the left adrenal vein for determination of pre-stress adrenal ascorbic acid levels. After a total collection period of adrenal vein effluent for 1 hour, the left adrenal was removed and analyzed for post-stress adrenal ascorbic acid levels. Analyses of adrenal ascorbic acid were made by the method of Roe and Kuether (9). Corticosterone in adrenal venous blood was extracted, chromatographed and determined photometrically as described earlier (10). Testes, seminal vesicles, prostates and thyroids were removed and weighed immediately following removal of the second adrenal. Tissues were fixed in Bouin's solution and sections from several areas of each tissue were processed by staining with Mallory's stain or hematoxylin and eosin. Brains were removed leaving the base of the skull attached to the ventral portion and were placed in 10% formalin. After decalcification of the skull, the brains were processed and the paraffin sections cut serially at 20 $\mu$ and stained with thionine. Reconstructions of each brain consisted of mapping on graph paper the sagittal relationship of areas represented by each ninth section at a magnification of 10.

## RESULTS

Table 1 shows the relationship between adrenal ascorbic acid decrease and corticosterone release into the adrenal venous effluent of the rat following the stresses of laparotomy and cannulation when large lesions had been placed in the hypothalamus.

Animals with hypothalamic lesions were divided into 4 major groups (B–E) on the basis of the adrenal response to stress. These groups were further subdivided, as shown in the table, on the basis of variations in the weights of target organs normally under the influence of pituitary hor-

[2] DC Labtronics Power Supply.

[3] Electrodes were bared up to 0.5 mm. from the tip.

TABLE 1. EFFECT OF HYPOTHALAMIC LESIONS ON ADRENAL ASCORBIC ACID DECLINE, CORTICOSTERONE RELEASE AND ORGAN WEIGHTS

| Group | No. of rats | Adrenal ascorbic acid, mg.% | | Corticosterone release | | Organ weights | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Pre-cannulation | % change | Total blood, ml. | μg./hr. | Testes, gm. | Sem. ves., mg. | Prostate, mg. | Thyroid, mg. | Adrenal, mg. |
| A<br>*Non-lesioned*<br>Normal ascorbic acid and corticoids | 9 | 430 ± 12* (9) | −34 ± 2 | 4.8 ± 0.4 | 52 ± 4 | 3.64 ± 0.20 | 277 ± 17 | 221 ± 20 | 12.0 ± 0.9 | 43.0 ± 1.7 |
| B<br>*Lesioned*<br>Normal ascorbic acid and corticoids | 7 | 391 ± 25 (37) | −34 ± 3 | 4.4 ± 0.5 | 53 ± 6 | 1.18 ± 0.25 | 61 ± 14 | 43 ± 10 | 12.1 ± 0.6 | 37.7 ± 2.2 |
| | 3 | | −31<br>(−29, −30, −33)† | 2.4<br>(2.2, 2.3, 2.6) | 43<br>(39, 41, 49) | 2.48<br>(2.32, 2.45, 2.68) | 78<br>(60, 65, 108) | 39<br>(28, 30, 60) | 8.9<br>(8.6, 8.7, 9.3) | 39.9<br>(35.9, 40.2, 43.7) |
| | 2 | | −30<br>(−29, −30) | 3.5<br>(3.0, 3.9) | 40<br>(38, 42) | 1.17<br>(1.03, 1.30) | 60<br>(55, 64) | 38<br>(30, 46) | 11.9<br>(11.2, 12.5) | 27.8<br>(26.6, 28.9) |
| | 25 | | −35 ± 1 | 4.3 ± 0.5 | 77 ± 6 | 3.56 ± 0.17 | 308 ± 16 | 202 ± 13 | 14.6 ± 0.3 | 50.8 ± 1.2 |
| C<br>*Lesioned*<br>Ascorbic acid inhibition | 3 | 354 ± 7 (5) | −6<br>(0, −8, −11) | 6.2<br>(4.9, 6.2, 7.5) | 73<br>(57, 61, 101) | 2.16<br>(1.24, 2.68, 2.55) | 90<br>(72, 98, 101) | 112<br>(98, 118, 120) | 13.2<br>(11.1, 13.9, 14.7) | 51.0<br>(46.4, 51.0, 55.5) |
| | 2 | | −11<br>(−10, −11) | 5.6<br>(5.0, 6.1) | 78<br>(61, 95) | 1.30<br>(0.66, 1.93) | 84<br>(59, 109) | 70<br>(37, 103) | 8.1<br>(8.0, 8.2) | 33.6<br>(33.3, 33.9) |
| D<br>*Lesioned*<br>Corticoid inhibition | 5 | 456 ± 31 (5) | −29 ± 4 | 4.2 ± 0.2 | 8 ± 1 | 3.16 ± 0.75 | 261 ± 16 | 212 ± 12 | 11.9 ± 0.2 | 45.9 ± 2.8 |
| E<br>*Lesioned*<br>Ascorbic acid and corticoid inhibition | 2 | 271 ± 22 (6) | −7<br>(−3, −10) | 5.6<br>(5.5, 5.7) | 5<br>(4, 6) | 1.34<br>(0.82, 1.86) | 101<br>(82, 120) | 89<br>(80, 98) | 14.0<br>(13.3, 14.7) | 44.9<br>(40.4, 49.4) |
| | 2 | | −2<br>(0, −3) | 4.4<br>(3.1, 5.7) | 5<br>(0, 10) | 0.86<br>(0.71, 1.00) | 60<br>(56, 53) | 31<br>(28, 33) | 8.5<br>(8.2, 8.7) | 43.4<br>(43.2, 43.6) |
| | 2 | | −7<br>(0, −13) | 3.5<br>(3.0, 3.9) | 12<br>(5, 19) | 1.67<br>(1.03, 2.30) | 60<br>(55, 64) | 38<br>(30, 46) | 11.4<br>(11.2, 11.5) | 27.8<br>(26.6, 28.9) |

* Standard error.
† Individual values.

mones. In Group B (37 rats) adrenal ascorbic acid decrease and corticosterone release were comparable to the responses observed in 9 control rats (Group A) subjected to the same stresses. On the other hand, in 5 rats (Group C) almost complete inhibition of adrenal ascorbic acid decrease occurred with control release levels of corticosterone. In 5 additional rats (Group D) a normal decrease in adrenal ascorbic acid occurred but with a minimal release of corticosterone. In 6 rats (Group E) both adrenal ascorbic acid decrease and corticosterone release appeared inhibited.

Pre-stress levels of adrenal ascorbic acid were essentially in the normal range (cf. Table 1) in all animals except those in Group E where severe damage to the median eminence and portal vessels was observed (*vide infra*). Resting levels of adrenal ascorbic acid in 5 animals similar to those in Group E but not subjected to adrenal vein cannulation were also very low ($257 \pm 19$ mg. %). The low levels of adrenal ascorbic acid in this group were therefore not a reflection of the stress of laparotomy.

No correlation was observed between the amount of blood collected from the adrenal vein and the corticosterone or ascorbic acid response to stress (Table 1).

Table 1 also depicts the relationship among the decrease in adrenal ascorbic acid, corticosterone release, and alteration in pituitary hormone target weights resulting from hypothalamic lesions. Inhibition of the decrease in adrenal ascorbic acid appeared coincident with gonadal atrophy in all cases (Table 1, Groups C and E). In 6 of these rats an additional thyroid and/or adrenal atrophy was present. On the other hand, specific inhibition of corticosterone release in 5 rats (Group D) occurred selectively since all of the target organs exhibited normal weights. The inhibition of both adrenal ascorbic acid decrease and corticosterone release (Group E) was non-selective in all cases, i.e. gonadal atrophy was present in all, thyroid atrophy in 2 rats and adrenal atrophy in 2 rats. On the other hand, normal adrenal ascorbic acid decrease and corticosterone release following stress in 12 lesioned rats (Group B) were coincidental with one or more atrophied target organs.

Figure 1 depicts the relationship between the average daily water intake, corticosterone release, and the per cent change in adrenal ascorbic acid. It appears that no correlation exists between water intake and either adrenal response.

From gross analyses based on the aggregate reconstruction of all brains from lesioned rats, lesions capable of inhibiting the normal decrease in adrenal ascorbic acid following stress without influencing corticosterone secretion (Table 1, Group C) appeared to be localized in the basal tuberal region of the hypothalamus. These lesions extended from the anterior hypothalamus to the anterior border of the mammillary body (Fig. 2). In most instances where both adrenal responses were inhibited, and altered target organ weights also noted (Table 1, Group E), the median eminence

and stalk exhibited either direct damage (but not total) or the lesions themselves effected an isolation of the median eminence in posterior and mid-regions. The median eminence appeared hypertrophied in most instances and in many cases hyalination was present in the anterior pituitary. It should be re-emphasized that in no instance was a specific inhibition of the adrenal ascorbic acid response to stress observed (i.e., without concomitant target organ atrophy) (Table 1).

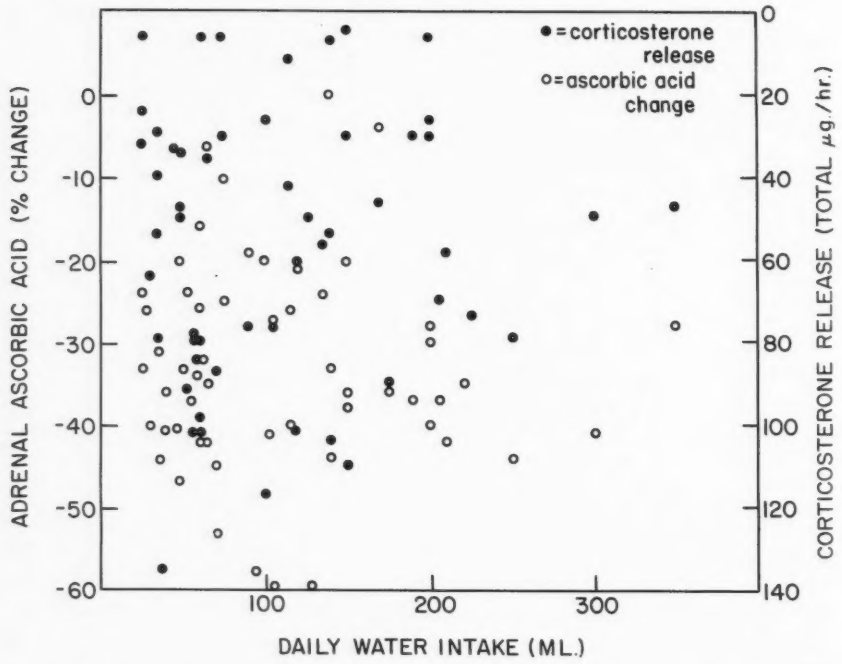


FIG. 1. Adrenal response to stress in rats with hypothalamic lesions as a function of water intake. Each point represents the decrease in adrenal ascorbic acid or the release of corticosterone plotted against the average daily water intake in a single rat for the 3 week period following operation.

In contrast to the area related to inhibition of adrenal ascorbic acid decrease, lesions *specifically* inhibiting the stress-induced release of corticosterone (Table 1, Group D) involved the posterior and mid-central portion of the hypothalamus (Fig. 2). These lesions did not include the anterior or medial portion of the median eminence region and in most cases extended dorsally as far as the massa intermedia. It would appear from Figure 2 that the cross-hatched lesioned area would result in the inhibition of both adrenal ascorbic acid decrease and corticosterone release. However, lesions in this area which inhibited corticosterone release were actually laterally

placed as well as being accompanied by more dorsal hypothalamic damage than the central basal tuberal lesions observed with adrenal ascorbic acid decrease. Previous investigations in our laboratory (11) have indicated that damage in the posterior basal tuberal region inhibited adrenal ascorbic acid decrease.

## DISCUSSION

Large lesions in the hypothalamus have been found capable of selectively inhibiting the decrease of adrenal ascorbic acid in response to the stresses of laparotomy and cannulation without altering the normal stress-induced release of corticosterone into the adrenal venous effluent of the rat.

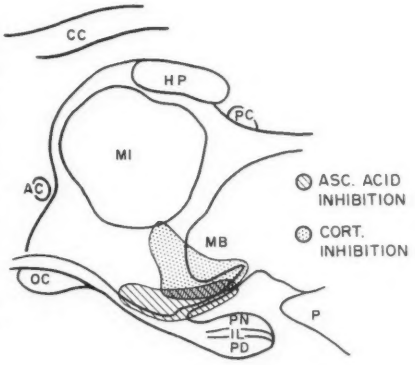


FIG. 2. Location of hypothalamic lesions associated with the inhibition of adrenal ascorbic acid decrease or corticosterone release following stress in the rat. A sagittal reconstruction of the hypothalamus and adjoining areas is shown. The following abbreviations are employed: AC: anterior commissure; CC: corpus callosum; HP: habenulo peduncular nucleus; IL: intermediate lobe of pituitary; MB: mammillary body; MI: massa intermedia; OC: optic chiasm; P: pons; PC: posterior commissure; PD: pars distalis; PN: pars neuralis.

Conversely, corticosterone release has been shown to be inhibited following certain hypothalamic lesions with no alteration in the adrenal ascorbic acid response. In some instances, both adrenal responses appeared inhibited. It should be pointed out, however, that *only* lesions inhibiting corticosterone release *per se* appeared to be specific, inasmuch as no alteration in any target organ weight dependent on other pituitary hormones was noted in this circumstance.

This work confirms previous investigations relating the maintenance of the target endocrine organs to specific hypothalamic areas. Those lesions found to involve the anterior portion of the median eminence in the rat were effective in producing adrenal atrophy in confirmation of the reports by Greer (12, 13), Bogdanove and Halmi (14) and Bogdanove (15). At variance with these results are those of McCann (1) and Ganong and Hume (16) who found that lesions in the median eminence apparently capable of inhibiting ACTH release did not prevent the maintenance of adrenal weight in the rat (1) and dog (16). In the present work, lesions in the anterior hypothalamus, just below the paraventricular nucleus and ventral in a midline section to the median eminence, resulted in thyroid atrophy. This area has been reported by Greer (12, 13), Greer and Erwin (17, 18),

Bogdanove and Halmi (14) and Bogdanove (15) to be required for the influence of goitrogenic agents on the rat thyroid. Testes and sex accessory weight maintenance appeared to be dependent on an intact area involving the lateral and posterior portions of the ventral medial and arcuate nuclei of the hypothalamus. This area is identical with that required for gonadal maintenance in male rats according to McCann (1), Bogdanove and Halmi (14), Bogdanove (15) and Harris (19). It would appear from the present studies that those areas concerned with maintenance of target organ weights are distinct from those involved in adrenal stress mechanisms (Table 1, Group B), inasmuch as 12 rats exhibited alterations in one or more target organ weights with normal adrenal responses.

McCann and Brobeck (7) reported a correlation between high water intake and a block in adrenal ascorbic acid decrease following the stress of laparotomy in the lesioned rat. Greer and Erwin (18) did not find such a correlation in rats exhibiting high water intake and amphenone-induced adrenal hypertrophy. In the present studies, no correlation between high water intake and inhibition of either adrenal response to stress was noted. Although the antidiuretic hormone may play a role in the stress phenomenon, its release does not appear to be directly correlated with pituitary-adrenal activation, as measured by either index employed in these studies.

Most investigators have emphasized the necessity of an intact hypothalamic-hypophyseal tract for the stress-induced release of ACTH. In our experiments lesions in the posterior hypothalamus not directly involving the median eminence were capable of inhibiting the release of adrenal corticosterone in response to stress. On the other hand even partial damage to the median eminence appeared to prevent the normal decrease in adrenal ascorbic acid following the stress of laparotomy. Hence it would appear that an intact hypothalamic-hypophyseal tract is more essential for adrenal ascorbic acid decrease than for corticosterone release in the rat.

Anderson, *et al.* (20) have implicated a mesencephalic-hypothalamic-pituitary activating system in the ultimate increase in urinary corticoids following stress. On the basis of neurological studies by Nauta and Kuypers (cf. Anderson, *et al.* (20)) it would appear that ascending mesencephalic pathways send collaterals into the hypothalamus. In our experiments, as long as any portion of the hypothalamic-hypophyseal pathway was intact and portal vessel damage was not total, corticosterone release following stress was still possible. On this basis it may be suggested that hypothalamic collaterals are numerous permitting hypothalamic-hypophyseal activation of ACTH release under these conditions. Specificity of the lesioned areas associated with the two adrenal responses in the present studies should be viewed with caution in view of the grossness of the lesions as well as the relatively small number of animals in each group.

The experiments reported above indicate that the release of cortico-

sterone into the adrenal venous effluent following stress does not appear to depend upon a concomitant depletion of adrenal ascorbic acid. The explanation for this dichotomy is not apparent, but it is possible that these discriminatory responses are a result of threshold differences in the response of the adrenal to circulating levels of ACTH, or that factors other than pituitary-adrenocortical activity are involved in the adrenal ascorbic acid response. These observations could also be explained by the existence of two separate pituitary adrenocorticotropic factors controlled by different hypothalamic pathways. However, evidence from numerous other lines of investigation would not appear to support this hypothesis (21).

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# ON THE INDUCTION OF MOLT IN BIRDS BY 17$\alpha$-OXYPROGESTERONE-17-CAPRONATE

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ABSTRACT

The effect of 17$\alpha$-oxyprogesterone-17-capronate on molting of 19 species of birds was investigated. Birds fall into two separate groups with regard to their response to the hormone. Some species molt after injection and others do not. The birds of the first group are those which breed the year round, and the second group have a restricted breeding season. The latter are mostly wild species. Bengalees and canaries, respectively, in our tests were representative of each group.

Oxyprogesterone capronate does not accelerate the growth rate of feather regeneration in either type of bird, but, rather, it inhibits it in canaries. The hormone seems to stimulate the resting papillae to become active in the birds of the first group and it can induce molt of the feathers of tail, wing and body. In Bengalees molt begins about one week after injection regardless of the amount of the hormone. The duration of molting, and the numbers of cast-feathers are proportional to the dose from 1.25 to 6.25 mg. There is no sexual difference in the molting response to the hormone. Gonadectomy does not induce molt nor does it make any difference in molting response by Bengalees to oxyprogesterone capronate.

Complete thyroidectomy in Bengalees inhibits the molt-inducing action of oxyprogesterone capronate, but partly thyroidectomized birds respond by molting. The molt is also induced by the hormone in birds treated with thiourea, but in this case the molting is delayed and prolonged.

Oxyprogesterone capronate does not cause any significant histological changes in the thyroid glands in Bengalees. Desoxycorticosterone acetate, estradiol benzoate and testosterone propionate do not evoke molt either in Bengalees or canaries.

From the results obtained, it is postulated that in Bengalees, oxyprogesterone capronate can induce molt by either 1) enhancement of sensitivity of feather papillae to thyroid hormone which can induce molt alone, or 2) synergistic action between oxyprogesterone capronate and thyroid hormone, or possibly a combination of those two.

IT HAS been shown in canaries, that molting is inhibited completely by both estrogens and androgens, but is not affected by either progesterone or desoxycorticosterone acetate (1). Recently, Shaffner (2, 3, 4) and Adams (5, 6) have reported that progesterone can induce molt in hens at any season of the year. Juhn and Harris (7) have demonstrated that progesterone injected intradermally into the immediate vicinity of the resting and quiescent papillae, does not affect them. However, it may exert a slight aug-

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TABLE 1. EFFECT OF OXYPROGESTERONE CAPRONATE ON MOLT IN BIRDS

| Species | Sex used | Doses* of the hormone injected (mg.) |
|---|---|---|
| **Birds which responded to PC by molting** | | |
| Psittacidae | | |
| 1. *Melopsittacus undulatus* | F, M | 6.25, 12.5, 18.8 |
| Ploceidae | | |
| 2. *Uroloncha striata* var. *domestica* | F, M | 1.25∼12.5 |
| Phasianidae | | |
| 3. *Coturnix coturnix japonica* | F, M | 18.8 |
| **Birds which did not respond to PC by molting** | | |
| Fringillidae | | |
| 4. *Passer montanus saturatus* | F, M | 6.25 |
| 5. *Eophona personata personata* | F, M | 12.5, 25 |
| 6. *Pyrrhula pyrrhula griseiventris* | F, M | 8.4, 12.5 |
| 7. *Emberiza cioides ciopsis* | F, M | 6.25, 12.5 |
| 8. *Emberiza spodocephala personata* | M | 6.25 |
| 9. *Emberiza rustica latifascia* | F, M | 12.5 |
| 10. *Carduelis spinus* | M | 6.25 |
| 11. *Uragus sibiricus sanguinolentus* | F, M | 6.25, 12.5 |
| 12. *Serinus canarius** | F, M | 3.2∼12.5 |
| Alaudidae | | |
| 13. *Alauda arvensis japonica* | M | 12.5 |
| 14. *Zosterops palpebrosa japonica* | M | 6.25 |
| Paridae | | |
| 15. *Parus varius varius* | M | 6.25 |
| Ploceidae | | |
| 16. *Taeniopygia castanotis* | F, M | 6.25, 12.5 |
| 17. *Padda oryzivora* | F, M | 9.5 |
| Timaliidae | | |
| 18. *Leiothrix lutea lutea* | F, M | 12.5 |
| Columbidae | | |
| 19. *Columba livia* var. *domestica* | F, M | 25 |

* The birds received single injection, except canary, in which the injection was repeated seven or eight times.

mentation on feather regeneration from the plucked stimulated papillae. Furthermore, they have shown that thyroxine is effective in causing regeneration of feathers in resting, quiescent and plucked papillae. Thus, the action of progesterone may not be the same on molting in all types of birds. This investigation was undertaken to obtain further information on this point.

## MATERIALS AND METHODS

As subjects of study, 19 species of birds were used, these are listed in Table 1. Of these birds, canaries (*Serinus canarius*) and Bengalees (*Uroloncha striata* var. *domestica*) were studied in greater detail than the other birds, because of their ready availability throughout the year. 17α-oxyprogesterone-17-capronate (PC)[1] was used and injected

[1] The symbol PC when used in this paper, refers to 17α-oxyprogesterone-17-capronate (Proluton-Depot, Schering A. G. Berlin).

into breast muscle of the experimental birds usually in a volume of 0.1 cc. Control birds received the same volume of sesame oil. As a measure of the molting process, the number of feathers cast off by the birds in a cage was counted and each bird carefully examined at the same time. The number of birds kept in a cage did not exceed six. They were subjected to natural daylight and allowed to take water and food *ad libitum*. They were kept at room temperature, unless otherwise stated. Room temperature in Japan varies from about 10° C in winter to about 30° C in summer. Other details concerning caging and techniques employed have been described in a previous paper (8), but some will be described below whenever necessary.

## RESULTS

### *Experiments with Canaries*

*Effect of oxyprogesterone capronate on molt* On October 21, six adult female birds were divided into two Groups A and B. Three birds of Group A were renewing the body feathers and also second or third primary feather counted from the outside of the wing, and those of Group B had finished completely the molt of their wing feathers, but were renewing body feathers. The details of the molting process of the canary have been described in a previous paper (9). Beginning on October 27, all the birds received 6.25 mg. of PC every fourth day for seven injections in all. Observations were continued over the following 8 days. Molting was not appreciably affected by the injections in either group of birds, and it terminated normally in both groups about the middle of November. The same treatment with PC failed to accelerate molting of canaries in July, when they were at the beginning of the annual normal molting period. Beginning on October 21, three canaries, which had completed their normal molt were injected with 12.5 mg. of PC in the same manner as in the previous experiment. Molt was not induced by this treatment during the 37 days of observation. The results were the same when the experiment was carried out in the non-molting period between December and March of the next year.

In the next experiment young birds with juvenile feathers, which hatched in early summer of the year were used. On December 31, three young canaries received a single injection of 9.5 mg. of PC, but, as in the adult, they did not molt in response to the hormone, although the observation was continued for 31 days until January 31. Thus, PC did not induce molt in either adult or young canaries in any season of the year.

*Effect of oxyprogesterone capronate on feather regeneration.* Shaffner (2) has reported that progesterone-treated domestic fowl showed full feather regeneration from the defeathered papillae, while non-treated ones failed to regenerate. This effect of progesterone was examined in the canary. In the non-breeding season, November 27, adult canaries with completely renewed feathers were divided into three Groups A, B and C, consisting of four birds each. On the same day, the feathers of the right half of the breast and the third primary feather of the left wing counted from the

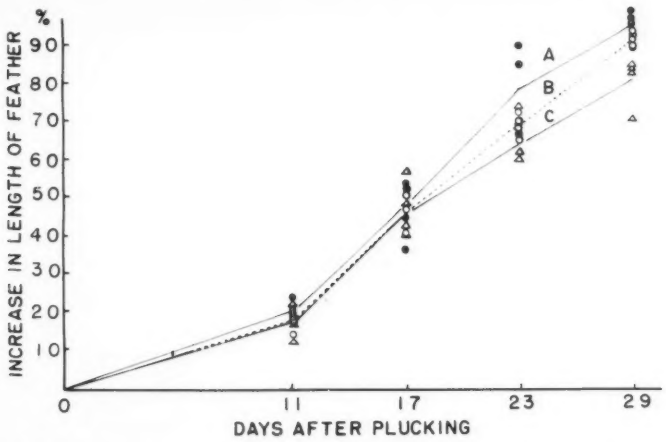


FIG. 1. Effect of oxyprogesterone capronate on regeneration of canary breast feathers. The symbols are expressed as percent increase of original length. Each value is an average of three feathers of one bird. ●—● Control birds (Group A); ○—○ Birds receiving 3.2 mg. of PC (Group B); △—△ Birds receiving 6.25 mg. of PC (Group C).

outside of the wing were plucked in all birds. Beginning on the day of the plucking, the birds of Groups B and C received 8 injections of PC. The injection was performed every fourth day. The dosage of each injection was 3.2 mg. in Group B and 6.3 mg. in Group C. The birds of Group A, received sesame oil, and served as controls. The observations were carried out for 30 days and the results are shown in Figures 1 and 2. Regeneration

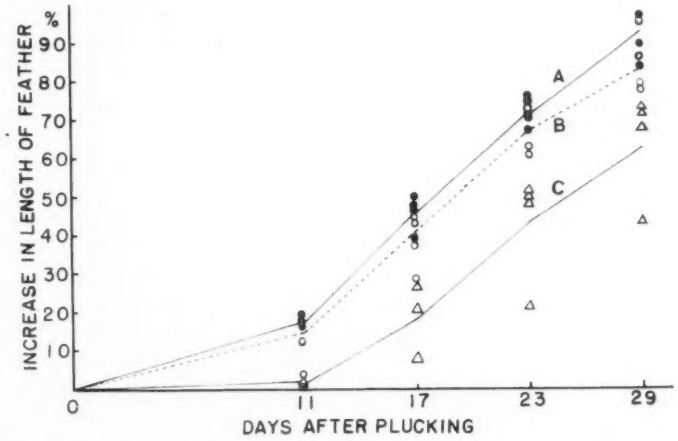


FIG. 2. Effect of oxyprogesterone capronate on regeneration of canary primary feathers. Each point represents percent increase in length of the third primary feather of one bird. The symbols are the same as in Figure 1.

of both types of feathers took place in all groups. The rate of growth o feathers was decreased by PC, compared with control birds, especially i the wing feather of the birds injected with high doses of the hormone.

*Experiments with Bengalees*

*Preliminary experiment.* Six birds were injected with a single dose o 6.25 mg. of PC on December 20, and six other birds receiving sesame oi served as controls. These animals were observed for a period of 50 days. I was found in this species that PC was capable of inducing molt in tail wing and body feathers. The molt began 8 days after the injection and i continued about 12 days.

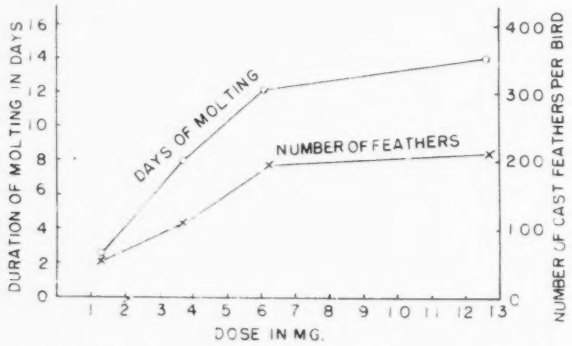


FIG. 3. Relation between dosage of oxyprogesterone capronate and response in molt in Bengalees.

*Relationship between dosage and response.* The molting response of Bengalees to graded dosages of 0.25 to 12.5 mg. of PC was observed in groups of 3–6 birds given each dose. The experiment was performed from December to March of the next year. Single injections of less than 1.0 mg. of the hormone were not effective, but amounts above 1.25 mg. induced the molt of all types of feathers. Regardless of the amount of the hormone, the molt always began about a week after injection. The peak of feather drop occured 11 to 13 days after the injection. The complete data are summarized in Figure 3. This figure shows the number of cast feathers and the duration of molting induced by varying dosages of PC. The duration of molting and the number of cast feathers were proportional to the dose from 1.25 to 6.25 mg. of PC.

*Effect of sex on molt induced by oxyprogesterone capronate.* Three females and three males were given single injections of 3.75 mg. of PC on February 2. They showed almost the same response in molt; thus, no sexual difference was observed (Fig. 4). To determine what influence the gonads might have on molting, seven adult males and two adult females were castrated

The female Bengalees like the domestic hen can lay eggs at any season of the year. Gonadectomy did not induce molt during the subsequent months. Therefore, it may be assumed that the PC induced-molt is not caused by a decrease in gonadal activity at least in this species. A single injection of 3.1 mg. of PC into these gonadectomized birds caused them to molt 9 days later, and the molting continued for seven days. Thus, the action of this hormone on molt in gonadectomized birds was almost the same as in normal intact animals (cf. Fig. 3).

*Effect of thyroidectomy and thiourea on oxyprogesterone capronate-induced molt in Bengalees.* On February 23, six birds were thyroidectomized (8), and 7 birds were partially thyroidectomized and 7 other birds were sham-

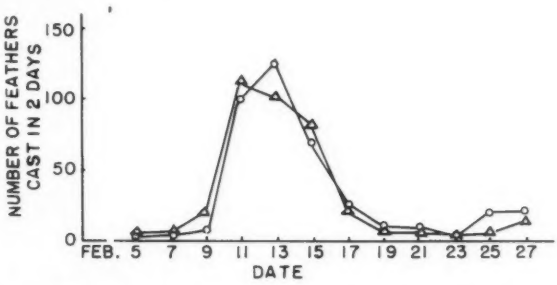


FIG. 4. Effect of sex on molt induced by oxyprogesterone capronate in Bengalees. The symbols represent the number of cast feathers by three birds for every 2 days. △—△ Male Bengalees receiving 3.75 mg. of PC; ○—○ Female Bengalees receiving 3.75 mg. of PC.

operated. They were kept individually at 22° C. The next day, they received 3.7 mg. of PC. Observations were made for 26 days in all. In the thyroidectomized birds, molt was not induced by the injection of the hormone. The largest number of feathers shed by an individual bird in any two day period was nine feathers. However, in 7 birds only partially thyroidectomized and in 7 sham-operated birds molt was induced by PC (3.7 mg.) about a week after the injection. The molting continued for eight days in both groups, but the numbers of cast feathers of both sham-operated (50 feathers per bird for 8 days) and partially thyroidectomized birds (80 feathers per bird for 8 days) were somewhat less than the number of cast feathers of intact PC treated birds (110 per bird for 8 days) kept at 13° C. It is not clear whether the decrease in cast feathers of the operated and sham-operated birds is due to the operation or to the differences in environmental temperature. At any rate, it seems that temperature does not affect seriously the action of PC on molt.

Beginning on February 16, seven birds kept at room temperature (13° C) were given 1% of thiourea in the drinking water. Since five of them died within 7 days, the concentration of thiourea was decreased to 0.25% begin-

ning February 23. On that day, 3.1 mg. of PC was injected into the two remaining birds. These began to respond to the hormone a week after the injection (Fig. 5). The number of cast feathers was greater and the duration of molting was longer than for the birds treated with PC alone (cf. Figs. 3, 4).

It may be concluded that molt is induced by progesterone capronate even when thyroid hormone is reduced, but it is not induced when the thyroid hormone is absent as following complete thyroidectomy.

*Histology of thyroid glands of the birds injected with oxyprogesterone capronate.* The birds injected with single dose of 3.7 mg. of PC were sacrificed at

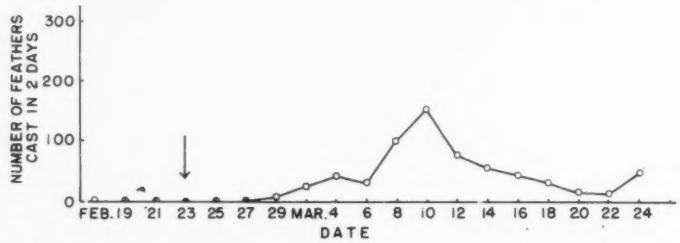


FIG. 5. Effect of thiourea on molt induced by oxyprogesterone capronate in Bengalees. The symbols show the number of feathers cast by 2 birds for every 2 days. Arrow indicates the day of PC injection.

various intervals of time. The cell height of the epithelium of 10 follicles in a section through the middle portion of the thyroid was measured. The mean cell heights with standard errors were $5.5 \pm 1.25$, $4.5 \pm 1.03$, $7.0 \pm 1.08$ and $5.3 \pm 0.75\mu$ at 0, 3, 6 and 12 days after the injection, respectively. In each group five birds were used. There was no significant difference between the groups of birds killed at any interval. Therefore, it would appear unlikely that the molt induced by PC is ascribable to the activation of thyroid glands by the steroid.

*Induction of molt by thyroxine in Bengalees.* To compare the mode of action of PC with that of thyroxine, single doses of 0.05, 0.1, 0.16, 0.2, 0.4 or 0.6 mg. of DL-thyroxine dissolved in slightly alkaline physiological saline solution were injected into five Bengalees, respectively. The feathers cast every day were counted and their numbers were plotted in Figure 6. The minimal effective dose was 0.2 mg. The feathers began to fall three days after the injection, and the duration of feathering was about one week for the dosages above 0.2 mg. of thyroxine. Feather loss was roughly proportional to the dose of thyroxine. The birds injected with 1 mg. of thyroxine shed all their feathers and became almost naked. Thus, thyroxine-induced molting appears different from that induced by PC treatment.

*Effect of oxyprogesterone capronate on feather regeneration.* On February 16, right breast feathers were plucked in five birds and they received two

injections of 1.25 mg. of PC, one on the same day, and one 13 days after the plucking. Five other birds, injected with sesame oil, whose feathers were plucked from the same area, served as controls. In three of five control birds, regeneration was sporadic. In these birds only few follicles showed regeneration, and feather germs did not appear in most defeathered follicles even in April when the observation was terminated. In two remaining controls the regeneration was almost as rapid as in the experimental birds. Experimental birds showed full feather regeneration in the defeathered area of the right breast within the period of observation. However, the growth rate of regenerating feathers was almost the same in the

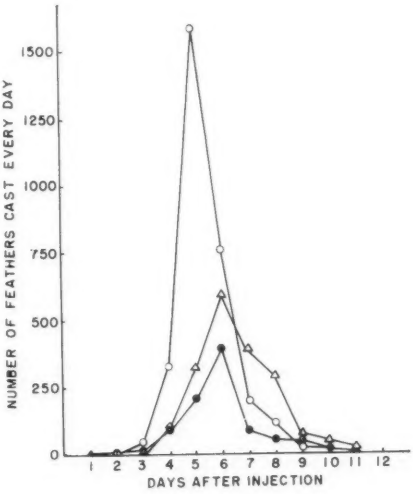


FIG. 6. Effect of thyroxine on molt in Bengalees. ●—● Five birds receiving 0.2 mg. of thyroxine; △—△ Five birds receiving 0.4 mg. of thyroxine; ○—○ Five birds receiving 0.6 mg. of thyroxine.

experimental birds as in two control birds which showed feather regeneration. For instance, 22 days after the beginning of the experiment, the mean lengths of three growing feathers in individual treated birds were 1.1, 1.3, 1.1, 1.2, 1.1 cm. in experimental birds and 1.2, 1.2 cm. in two of the five controls.

*Effects of other steroids.* Single injection of desoxycorticosterone acetate (DCA, 2.5 mg.), estradiol benzoate (0.1 mg.) and testosterone propionate (0.5 mg.), which were dissolved in sesame oil, did not induce molt in either canaries or Bengalees.

### SPECIES DIFFERENCE IN RESPONSE OF MOLT TO OXYPROGESTERONE CAPRONATE

As is shown in Table 1, molt was induced by a single injection of PC, ranging from 6.25 to 18.8 mg. in amount, in Bengalees, Japanese quails (*Coturnix coturnix japonica*) and parakeets (*Melopsittacus undulatus*).

There were some differences in response among them (Fig. 7). However, no molt was induced in 16 other species of birds by the administration of the hormone.

## DISCUSSION

It has been shown in the experiments reported here that birds fall into two groups with regard to the response of their feathering to progesterone capronate: 1) those species in which the hormone can induce molt, and 2)

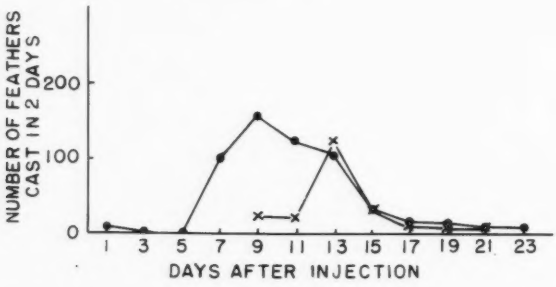


FIG. 7. Effect of a single injection of oxyprogesterone capronate on molt in parakeets (*Melopsittacus undulatus*) and Japaneses quails (*Coturnix coturnix japonica*). ●—● Two parakeets (♂, ♀) injected with 12.5 mg. of PC; ×—× Three Japanese quails (♀, ♀, ♂) injected with 18.8 mg. of PC.

those which do not respond to the hormone by molting (Table 1). The birds belonging to the first type have as a common characteristic the year-around laying of eggs. In these birds there was no sexual difference in response to the hormone. The second group includes mostly wild species which lay eggs only in restricted seasons usually in spring. Shaffner (4) states that progesterone can induce molt in pigeons, but this has not been confirmed in the present study. In later experiments, Harris and Schaffner (15) have likewise failed to induce molt of the pigeon by progesterone. The reasons PC can induce molt in domesticated continually breeding birds, and cannot induce it in the wild birds, are not clear now.

It is well known that estrogens and androgens inhibit molting of birds. Further, it has been shown recently that progesterone also inhibits the egg production in domestic hens (2, 3, 5, 6, 7) and inhibits light-induced development of the ovary in canaries (11). From these results, there is a possibility that decrease of the concentration of estrogens or androgens in the circulating blood owing to progesterone induced gonadal inactivation, may lead the birds of Type 1 to molt. The validity of this conclusion is cast in doubt by the present study, in which it has been found that molt is not induced by gonadectomy in Bengalees. Therefore, decrease of gonadal hormones in the blood may not be a principal cause of molt induction in this species. Yet, it seems likely that it permits the birds to molt more easily

Here, we must suppose that the primary molt-inducing action is a property of PC itself in Bengalees. However, as is mentioned below, in domestic hens, there is a possibility that a decrease of estrogens in blood may be a cause of molt by progesterone (12).

Failure of regeneration of the feathers in three control birds may be ascribed to inhibition by gonadal hormones. It is possible that the gonads may have been relatively inactive in the two other control birds, permitting regeneration to take place. Although in the canary experiment feather regeneration occurred in both experimental and control birds, the experiment differed, having been carried out in November. At that time canaries usually have inactive gonads (9), and so regeneration may have been free from the gonad hormonal influence. The occurrence of regeneration in all experimental Bengalees may have been partly due to gonadal atrophy which may have been caused by PC. But the motive force of the regeneration may have been the molt-inducing action of PC itself, since gonadectomy cannot induce molt. These results suggest that PC stimulates feather papillae, directly or indirectly, to regenerate new feather, since Wang (16) and Lillie and Wang (17) have demonstrated that only the mesodermal papilla can induce the feather proper in the epidermal cells covering its surface (18). The growth rate of regenerating feather was not accelerated by PC. Therefore, PC seems to work as a trigger for refeathering.

Thyroidectomy in Bengalees inhibited completely the effect of PC on molt. In the birds deprived of most of their thyroid tissue, or treated with thiourea, PC could induce molt. This suggests that PC cannot induce molt when the thyroid tissue is completely absent, but it can induce molt, if any thyroid tissue remains. From these results, we can suppose that the PC-induced molt is due to activation of thyroid tissue. Not in line with this idea is the fact that histologically, there is no significant difference between thyroids of normal birds and PC-injected birds. Therefore, it may be unlikely that molt is induced by activation of thyroid gland following the injection of PC. On the other hand, it appears impossible for the hormone alone to induce molting, since it does not cause the birds completely deprived of thyroid to molt. But thyroxine alone can induce molt in birds, although it does not have the same pattern of action as PC. Considering the results thus obtained, we can postulate two possibilities in relation to the mechanism of induction of molt by PC: 1) enhancement of sensitivity of feather papilla by PC to thyroid hormone, 2) synergistic action between PC and thyroid hormone. Either of these explanations, or both of them, may explain the molt induced by PC in thiourea-treated birds and the birds of which larger part of thyroid glands was removed. In the thiourea-treated birds, thyroid hormone may not be completely absent, since it has been demonstrated that in fowl treated with thiouracil protein-bound iodine is not reduced (10). Shaffner (3) has also observed that progesterone can induce molt in thiouracil-treated hens. If thyroid hormone intervenes in the

mechanism of induction of molt by PC, one question that may be raised is why the PC-induced molt does not occur as fast as that induced by a single injection of thyroxine. A possible reason may be that the amount of thyroid hormone in the birds injected with PC is smaller than that in those receiving a single injection of thyroxine. If the birds were injected daily with small amounts of thyroxine, which cannot induce molt by a single injection, their molt might take place later than the birds injected with single high doses of the hormone.

In the present study DCA, estradiol benzoate and testosterone propionate did not induce molt. But Juhn and Harris (7) have reported that DCA inhibits egg production and induces molt in domestic hens. This molt induced by DCA is supposed to be due chiefly to the decrease of estrogens in this species. Tanabe (12) could induce molt by starving hens and Pino (14) by feeding hens with Enheptin, and they found that at that time the hens had also inactive ovaries. Therefore, there is a possibility that in hens a decrease in estrogens in circulating blood may become a cause of molt. Juhn and Harris also have reported that testosterone injection induces molt, but that the testosterone-induced molt takes place later than that induced by progesterone and DCA. They observed that egg production in such hens was also inhibited. In this case also it seems likely that the molt was induced by decrease of estrogens in the body. The delay of beginning of the molt may be a result of the inhibitory effect of testosterone itself on refeathering.

*Acknowledgment*

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# A STUDY OF ADRENAL RADIOACTIVITY SUBSEQUENT TO PARENTERAL $Co^{60}$-LABELED VITAMIN $B_{12}$

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ABSTRACT

Data, gathered during a study on tissue distribution of parenterally administered radioactive vitamin $B_{12}$, show that the adrenal concentrates a relatively large amount of this radioactivity and that its ability to do so is markedly influenced by parenteral testosterone propionate.

Thirty-day-old littermate albino rats, consisting of testosterone-treated and control animals, received a single subcutaneous injection of 0.25 $\mu$g. $Co^{60}$-labeled vitamin $B_{12}$. With the exception of 6 animals, the rats received a subcutaneous daily injection of 16.6 $\mu$g. unlabelled vitamin $B_{12}$ from their 34th to 40th day of age; all rats were sacrificed on the 40th day.

The radioactivity of the ashed kidney, liver, adrenal, and thyroid tissues was measured.

The adrenal was found to assume a place among two of the tissues, the kidney and liver, which show the highest radioactivity concentration in the body; the thyroid contained a relatively insignificant amount of radioactivity. In addition it was observed that although the adrenal gland decreased in size following parenteral testosterone propionate, the absolute amount of radioactivity in the gland increased by more than 4-fold.

From these observations it is inferred that the radioactivity present in the adrenal gland, following the administration of labeled vitamin $B_{12}$, is intimately concerned with the activity of the gland.

IT IS the purpose of this paper to present evidence, gathered during a study on tissue distribution of parenterally administered radioactive vitamin $B_{12}$, indicating that the adrenal gland concentrates a relatively large amount of this radioactivity and that its ability to do so is markedly influenced by parenteral testosterone propionate.

## METHODS

Littermate rats (Carworth strain) were injected subcutaneously daily, from the day of birth, with either of two preparations: The control rats received 0.02 cc. of cottonseed oil; the experimental animals received 1.0 mg. of testosterone propionate[1] in 0.02 cc. of oil. From the 24th day of age through the completion of the experiment, the latter received 3 mg. of testosterone propionate while the controls received an equivalent volume of 0.06 cc. of oil. Injection sites were uniformly alternated so that no one site received the next injection until four days later. All subcutaneous injections were performed with a syringe controlled by a micrometer.

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[1] A cottonseed oil preparation kindly supplied by the Upjohn Company, Kalamazoo, Michigan.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE UPON THE RADIOACTIVE CONTENT OF THE ADRENALS SUBSEQUENT TO PARENTERAL $Co^{60}$-LABELED VITAMIN $B_{12}$ ADMINISTRATION

| Sex | Group | Number of rats | Body wt., gm. | Adrenal wt., mg./100 gm. body wt. | Adrenal radio-activity, cpm./100 mg. adrenal |
|---|---|---|---|---|---|
| Male | Control | 13<br>(2) | 100 ± 4<br>(80 ± 16) | 18.0 ± .6<br>(19.0 ± 3.4) | 97 ± 7<br>(95 ± 17) |
| | Testosterone-treated | 14 | 101 ± 4 | 14.5 ± .5* | 401 ± 26* |
| Female | Control | 5<br>(2) | 96 ± 5<br>(91 ± 20) | 20.0 ± 1.3<br>(25.0 ± 3.3) | 65 ± 23<br>(68 ± 2) |
| | Testosterone-treated | 5<br>(2) | 88 ± 3<br>(114 ± 3) | 14.0 ± 1.0*<br>(13.2 ± 1.5) | 385 ± 78*<br>(488 ± 119) |

( ) Denotes the data for animals that did not receive the unlabeled vitamin $B_{12}$.
± Standard error of the mean.
* Significantly different from control of the same sex ($P = 0.01$).

The rats were sexed at birth and only those of 1 sex were retained within a particular litter. Wherever possible the same number of rats was allotted to the control group as to the experimental group. The mothers, during pregnancy and lactation, as well as their offspring, were maintained on an all plant ration consisting of 35% yellow corn meal, 30% soybean meal (containing 50% protein), 25% ground whole wheat, 6% Alfalfa leaf meal, 3% Brewer's yeast (Strain G), and 1% iodized salt.

When 30 days old, each animal received a single subcutaneous injection of 0.25 μg. $Co^{60}$-labeled vitamin $B_{12}$.[2] With the exception of 6 animals, the rats received a daily subcutaneous injection of 16.6 μg. stable vitamin $B_{12}$[3] from their 34th to 40th day of age. All rats were sacrificed on the 40th day.

The animals were sacrificed in an ether chamber, then weighed. The adrenal and thyroid glands, and in a limited number of cases, the liver and kidney were rapidly removed, trimmed, and weighed, in tared planchets, to the nearest 0.1 mg. The tissues were dried in an oven at 100° C for 12 hours; then they were ashed at approximately 600° C for another 12 hours. Ash weights were determined, following the final temperature equilibration in a desiccator. Radiometric determinations of these samples were performed with a thin window G. M. tube and a laboratory scaler. Counts were corrected for self absorption by use of a predetermined curve. The specific activity of the $Co^{60}$-labeled vitamin $B_{12}$ preparation was 824 μc./mg. The total radioactivity administered was equivalent to 59,274 cpm. ± 0.9%. All samples were counted to the 2% level of accuracy, with the exception of 3 counts (5% level).

## RESULTS

The radioactivity collected by the adrenals per unit of tissue (Table 1) ranks between that of liver and kidney (Table 2). Data are not presented

[2] We are grateful to Merck & Co., Inc., Rahway, New Jersey for supplying the radioactive Vitamin $B_{12}$.

[3] A distilled water solution of a Merck's crystalline vitamin $B_{12}$ preparation (0.1% trituration with mannitol). As a preservative, phenol crystals were added to make a 0.5% solution.

on the thyroids because their activity was too low to provide significant counting rates.

Testosterone treatment resulted in a marked decrease in adrenal weights and increase in adrenal radioactivity in both males and females. In the major groups, receiving the "flushout" dose of non-radioactive vitamin $B_{12}$, the differences from the controls for the respective groups were highly significant ($P = 0.01$). Similar changes occurred in the testosterone-treated animals that did not receive additional vitamin $B_{12}$.

Both males and females treated with testosterone showed a tendency toward reduced radioactivity in kidney, but not in liver tissue.

## DISCUSSION

Evidence to date (1, 2, 3) indicates that the radioactivity found in tissues subsequent to administration of $Co^{60}$-labeled vitamin $B_{12}$ is due to the actual vitamin rather than cobalt ion or other cobalt-containing compounds. As indicated by the radioactivity measurements, the collection of vitamin $B_{12}$ in the adrenals of normal rats ranks between that of kidney and liver. The latter organs have been shown by Rosenblum *et al.* (4) to have high concentrations of radioactivity after administration of radioactive vitamin $B_{12}$.

The decrease in adrenal weight after dosage with testosterone propionate agrees with the report of Selye (5) on rats given similar treatment, and provides presumptive evidence of decreased function. The concurrent increase in vitamin $B_{12}$ retention could occur as a result of decreased utilization of the vitamin in metabolic processes of the gland as a result of its lowered activity. This, together with the relatively high affinity of the adrenal for radioactive $B_{12}$ suggests that this vitamin may play an important role in adrenal metabolism.

Becker *et al.* (6) observed a tendency, following parenteral testosterone

TABLE 2. RADIOACTIVE CONTENT OF THE KIDNEY AND LIVER SUBSEQUENT TO PARENTERAL $Co^{60}$-LABELED VITAMIN $B_{12}$

| Sex | Group | Body wt., gm. | Kidney | | Liver | |
|---|---|---|---|---|---|---|
| | | | gm./100 gm. body wt. | cpm./100 mg. kidney | gm./100 gm. body wt. | cpm./ 100 mg. liver |
| Male | Control (9) | 89 | 1.17 ± 0.038 | 574 ± 111 | 5.14 ± 0.229 | 80 ± 20 |
| | Testosterone-treated (10) | 98 | 1.19 ± 0.023 | 388 ± 47 | 5.08 ± 0.145 | 67 ± 10 |
| Female | Control (10) | 89 | 1.18 ± 0.028 | 626 ± 86 | 5.30 ± 0.241 | 75 ± 9 |
| | Testosterone-treated (9) | 87 | 1.33 ± 0.064 | 430 ± 45 | 4.72 ± 0.199 | 83 ± 5 |

± Standard error of the mean.
Number in parenthesis indicates number of animals in the group.

administration, toward a reduced vitamin $B_{12}$ content in the kidney and liver of female rats. The present data indicate a reduction in the kidney, but not the liver, of both males and females that were similarly treated.

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# SOME EFFECTS OF SEX HORMONES AND HOMOLOGOUS GONADOTROPHINS ON THE EARLY DEVELOPMENT OF THE RUDIMENTARY GONAD IN FOWL[1]

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ABSTRACT

The development of the right rudimentary gonad was studied in 477 untreated and hormone-treated brown Leghorns. In untreated sinistrally ovariectomized birds (poulards), the gonad was typically composed of medullary cords and tubules, but cortical tissue was found in 21.3%. Estrogen-treatment, started at the time of ovariectomy, had no effect upon the incidence or development of cortical tissue. In androgen-treated poulards the incidence of cortical tissue was reduced to 12.4%, but this reduction was not statistically significant. Both androgen and estrogen inhibited the growth and differentiation of medullary tissue in poulards. Estrogen-treatment resulted in vacuolization of the medullary cord cells and suppression of further development, whereas androgen caused a significant regression of the gonadal tissue.

The weights of pituitaries of hormone-treated poulards were significantly less than those of controls, and, in the older groups, this reduction in weight was significantly greater in estrogen-treated than in androgen-treated poulards. Treatment with chicken gonadotrophins, known to stimulate endocrine and gametogenic functions in the ovaries and testes of normal chicks, did not prevent the inhibitory effects of the steroid hormones in androgen and estrogen-treated poulards and failed to stimulate growth or function of the rudimentary gonad in normal poulards.

It is probable that the suppression of medullary tissue by the sex hormones is not due to pituitary inhibition and may be due to the direct action of these hormones on the gonad.

THE female domestic fowl normally has only one functional ovary, located on the left side. Although a right gonad is formed in the embryo, it soon ceases to develop and actually regresses. This right rudimentary gonad persists after hatching as a flattened ridge of tissue which may or may not be detectable grossly. It has frequently been stated and is generally accepted that the rudiment consists of medullary tissue and rarely contains a cortical component (see 1 for references). Domm (2) es-

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tablished that the removal of the functional left ovary usually results in the development of the rudimentary right gonad into a sterile testis-like organ, which can secrete both androgen and estrogen.

Previous work in this laboratory (1) as well as elsewhere (3) has shown that treatment with sex hormones inhibits the development of the rudimentary gonad after ovariectomy. Taber and Salley (1) implanted estrogen or androgen pellets into young pullets at the time they were ovariectomized. At autopsy, several months later, differentiation of medullary tissue had been completely inhibited, but well-developed cortical tissue was found in the right gonads of 11 of the 30 hormone-treated birds. No cortical tissue was found in the hypertrophied gonads of the 7 controls. Considering the reported rarity of cortical tissue in the rudimentary gonad, it was postulated that treatment with estrogen or androgen might have caused the formation and subsequent development of new cortical tissue. However, since sex hormones are not believed to originate a morphological component (4), it seemed more probable that cortical tissue had been present in these gonads at the time of ovariectomy. If so, its presence in the right gonad is not so rare as has been believed.

One purpose of the experiments reported in this paper was to determine the effects of androgen and estrogen-treatment on the early development and differentiation of the rudimentary gonad, since the previous work had described the effects observed after several months of hormone-treatment. It was hoped that such a study might help explain the mechanism of gonadal inhibition, and whether or not the inhibitory effects of androgen and estrogen are identical. Another purpose was to determine whether hormonal treatment stimulates the formation and development of cortical tissue or whether the incidence of this tissue is equally high in untreated right gonads.

## METHODS

Single-comb, light-brown Leghorn females were used in all experiments. In ovariectomized controls, increased vascularity and growth of the comb were used as indicators of androgen production by the right gonad. New feathers of the female-type served as an indicator of estrogen secretion. In birds implanted subcutaneously with androgen or estrogen pellets,[2] comb growth and plumage, respectively, served as indicators of the effectiveness of the exogenous hormonal stimuli.

All birds were ovariectomized at three weeks of age. At the time of autopsy the site of the left ovary was checked for regenerated fragments and, when possible, the right gonad was removed and weighed. If no gonadal tissue could be detected grossly, or if the tissue was spread diffusely along the inferior vena cava, the vein, Wolffian body and adjacent tissue were removed and, like the definitive gonads, serially sectioned and stained with hematoxylin and eosin.

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## EXPERIMENTS AND RESULTS

### *A. Right Gonads of Normal Pullets*

The rudimentary right gonads from 15 normal pullets, 3, 4, and 5 weeks old, were studied. The gonad in each of the three age groups was located in close proximity to the Wolffian body and, in histological sections, consisted of connective tissue strands separated by sinus-like spaces (Fig. 1). Within the strands were groups of medullary cord cells, the cytoplasm of which varied from granular to vacuolated (Fig. 9). In one of the 15 gonads cortical tissue was also present (Fig. 2).

### *B. The Development of the Right Gonad in Sinistrally Ovariectomized Birds (Poulards)*

Although the histological structure of the right gonad has been previously described (5, 6, 7), it seemed desirable to make a more detailed study of a larger number, particularly during the early stages of development, in order to interpret the changes produced by androgen or estrogen-treatment. Therefore, 118 pullets were sinistrally ovariectomized at three weeks of age and the right gonads were recovered 1, 2, 3, 4, 5, 6, 8, 10, and 28 weeks after ovariectomy.

The microscopic study of serial sections showed that the majority of these right gonads was composed entirely of medullary tissue, but cortical tissue was present in 26. In three birds, killed 3, 4, and 5 weeks after ovariectomy, no definite gonadal tissue could be identified.

Although there is considerable variation, it is evident from the study of this material that the development of the rudimentary gonad after ovariectomy can conveniently be described by dividing it into three periods: 1 to 2 weeks, period of cytological changes; 3 to 5 weeks, period of proliferation; after 5 weeks, period of differentiation.

One and two weeks following ovariectomy, the general microscopic structure of the right gonad did not differ significantly from that of normal pullets of the same age. However, there was a striking change in the medullary cord cells. These cells, called "type a" cords by Gray (6), were larger and filled with deeply staining granular cytoplasm (Fig. 10). "Fat-laden" cells, reported by Gray as scattered throughout the gonad, isolated or in cords, and sometimes comprising $\frac{1}{2}$ of the medullary cord cells, were rarely observed. Mitotic figures were seldom seen in medullary cells, but were abundant in cortical tissue.

The period from 3 to 5 weeks after ovariectomy was mainly one of active proliferation, indicated by the large number of mitotic figures, and the beginning of differentiation into small, sterile, medullary tubules. By the fifth week the gonad was usually large enough to be identified grossly with no difficulty. In histological sections, most of the sinus-like spaces previously observed appeared to be partially or completely obliterated by the

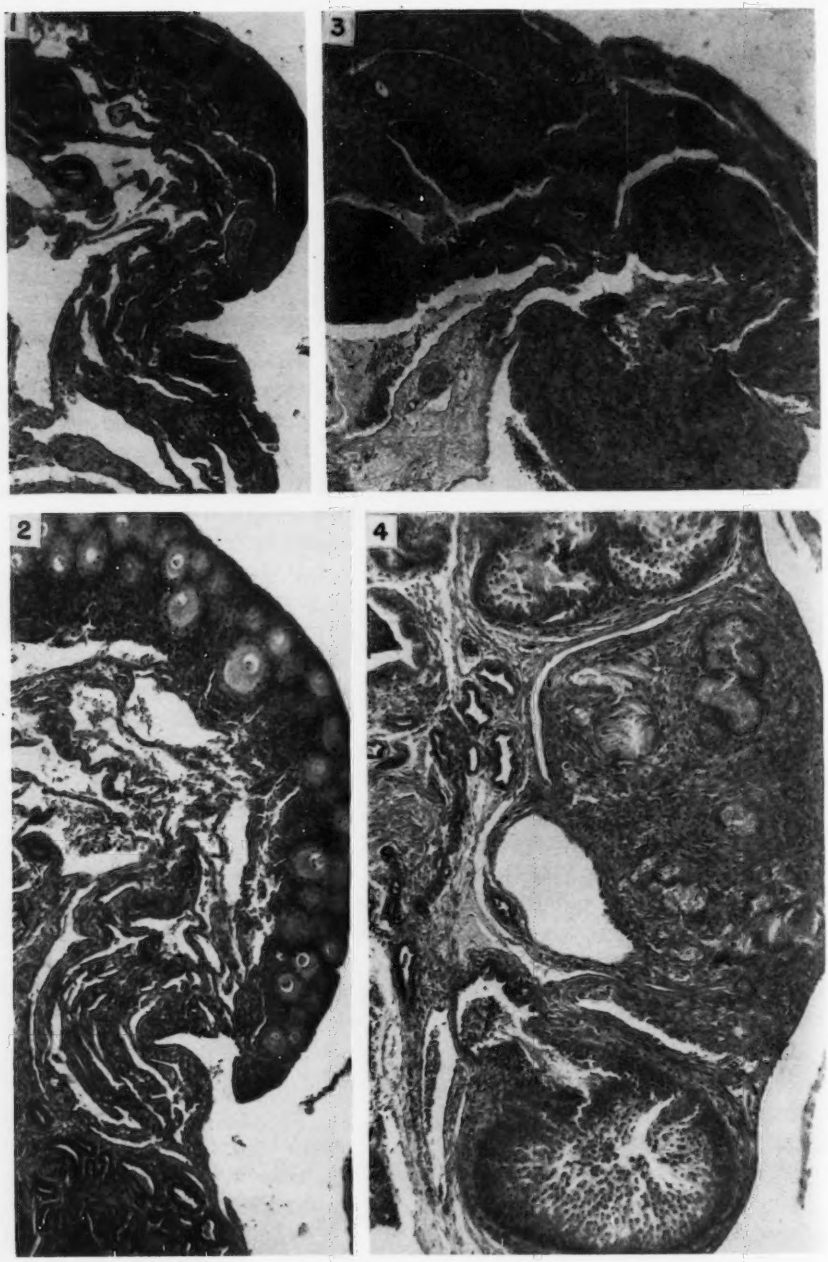


FIG. 1. Typical rudimentary gonad from a 4-weeks old pullet. The gonad consist of connective tissue strands separated by sinus-like spaces. ×100.

increased mass of medullary cells. The connective tissue remained and served to divide the gonad into small lobules, a characteristic feature of the fully developed gonad. Most of the cells were in definite cords and some were arranged as a single layer surrounded by a basement membrane (Fig. 3). Gray (6) called these "type b" cords and suggested that they represented the first stage in tubule formation. Benoit (7), however, described the origin of tubules from the proliferation of the lining of the sinus-like spaces ("lacunes"). Prior to proliferation the epithelium became columnar. In only three right gonads have we observed small patches of columnar cells such as those described and illustrated by Benoit, and in no case was there evidence that proliferation of these cells resulted in typical tubule formation.

Some gonads contained tubules with small lumina ("type c") which, except for the relative absence of primordial germ cells, resembled normal testicular tubules from a young chick. Some of the larger tubules resembled the sterile tubules of a mammalian cryptorchid testis; germinal cells were rarely present and the Sertoli cells formed a syncytium more or less filling the lumen. Such tubules ("type d") are typical of the right gonad of the sinistrally ovariectomized chicken at all later stages. Three right gonads, recovered 3 and 4 weeks after ovariectomy, contained small lobes of well-differentiated seminiferous tubules with cells undergoing spermatogenesis. In two of these, in addition to numerous primary and secondary spermatocytes, a few spermatids and an occasional spermatozoan were observed. These birds were 6 and 7 weeks of age. (Blivaiss (8) reported that the average age at which sperm are found in the testis of the brown Leghorn rooster is 18 to 20 weeks.) In both of these cases, as well as in older gonads containing well-developed fertile or sterile testicular tubules, connection between the tubules and the rete testis could be traced (Fig. 4). Gray (6) stated that he failed to observe any such connection. In these, as well as older gonads, it was noted that the tubules showing the highest degree of differentiation were usually located in close proximity to the Wolffian body. This suggests that the Wolffian body may play an inductive role in gonadal differentiation.

After 5 weeks there was a noticeable decrease in the number of mitotic figures and an increase in the degree of differentiation of the gonadal tissue. The gonads were typically lobular, the lobes consisting of sterile medullary

FIG. 2. Right gonad from a 4-weeks old pullet, containing cortex with follicles and medullary strands. Note the relation of the gonad to the Wolffian body. ×100.

FIG. 3. Part of rudimentary gonad 4 weeks after ovariectomy. Note increase in medullary tissue and a few small tubules. ×100.

FIG. 4. Part of rudimentary gonad 4 weeks after ovariectomy. Note well-developed seminiferous tubules with one connected to a rete tubule. Also note lobular nature of gonad, with sterile tubules in the middle lobe. ×100.

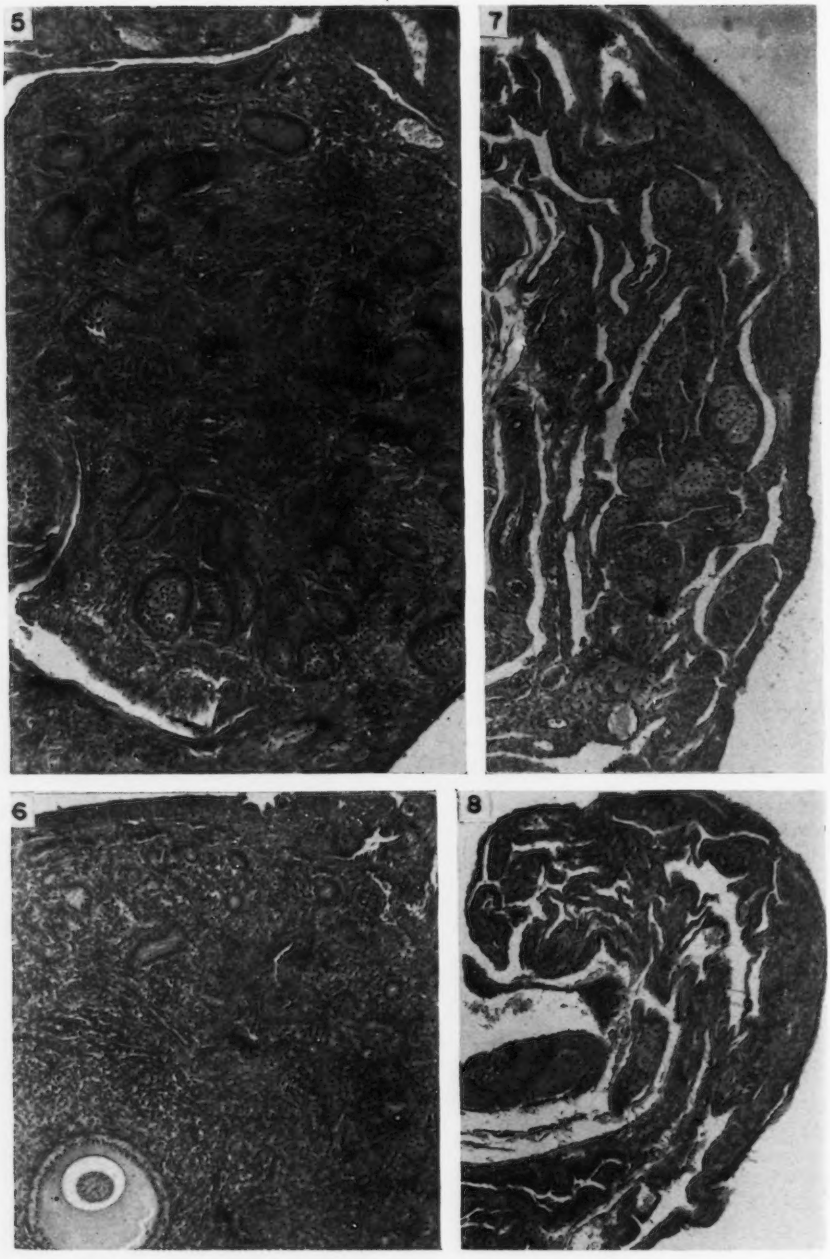


FIG. 5. Part of right gonad 10 weeks after ovariectomy. Note lobular structure and sterile and fertile tubules. ×100.

cords and "types c and d" tubules. Three gonads contained tubules showing active spermatogenesis (Fig. 5). By 8 weeks, the gonads were large and discrete enough to be weighed and averaged 49.2 mg. This increased to 64.2 mg. at 10 weeks and 562.2 mg. at 28 weeks.

Androgen-secretion, indicated by increased comb-vascularity and growth, was first detected in one bird 6 weeks after ovariectomy. The majority of the hypertrophied right gonads were producing androgen by 8 or 10 weeks and all by 17 weeks. There was no correlation between the degree of tubule differentiation in these gonads and androgen-secretion.

Cortical tissue was found in 26 gonads, including one or more from each age group except those killed 3 and 10 weeks after ovariectomy. Twelve of these were relatively normal ovaries. In the remaining 14, the amount of cortical tissue varied. In some both medullary and cortical components differentiated so that the gonads could be considered ovotestes (Fig. 6); in others cortical tissue was limited to one isolated follicle or to areas of sterile cortical cords.

The 12 birds with well-developed right ovaries had female plumage, indicating estrogen-secretion. In the remaining 14, some had female plumage and some had retained the male or neuter-type of feathers. Female plumage was observed in 6 other birds in which careful examination of serial sections of the gonads revealed no follicles and no cells which could be definitely identified as cortical cords.

The average weight of pituitaries from birds which contained identifiable cortical tissue was consistently less than that of the other birds of the same age. Because of the small numbers within each age group, these differences in pituitary weights were not tested for significance.

### *C. Effects of Avian Gonadotrophins on the Development of the Right Gonad*

Domm (9) and Kornfeld and Nalbandov (3) have shown that injections of mammalian gonadotrophic extracts had no effect upon the growth or function of the right gonad in sinistrally ovariectomized birds. Since then Das and Nalbandov (10) and Taber, *et al.* (11) have presented evidence supporting the hypothesis that there is a qualitative difference between mammalian and avian gonadotrophins. Therefore, it seemed possible that gonadotrophins from the chicken-pituitary might stimulate the rudimentary gonad, although mammalian gonadotrophins had failed to do so.

Nine birds were ovariectomized at three weeks of age and given daily (except Sunday) subcutaneous injections of approximately 13 mg. of pulverized, acetone-dried, chicken-anterior lobes suspended in saline. It was

FIG. 6. Part of right gonad 8 weeks after ovariectomy. Note follicle and small tubules. ×100.

FIG. 7. Right gonad from estrogen-treated bird 10 weeks after ovariectomy. ×100.

FIG. 8. Right gonad from androgen-treated bird 10 weeks after ovariectomy. ×100.

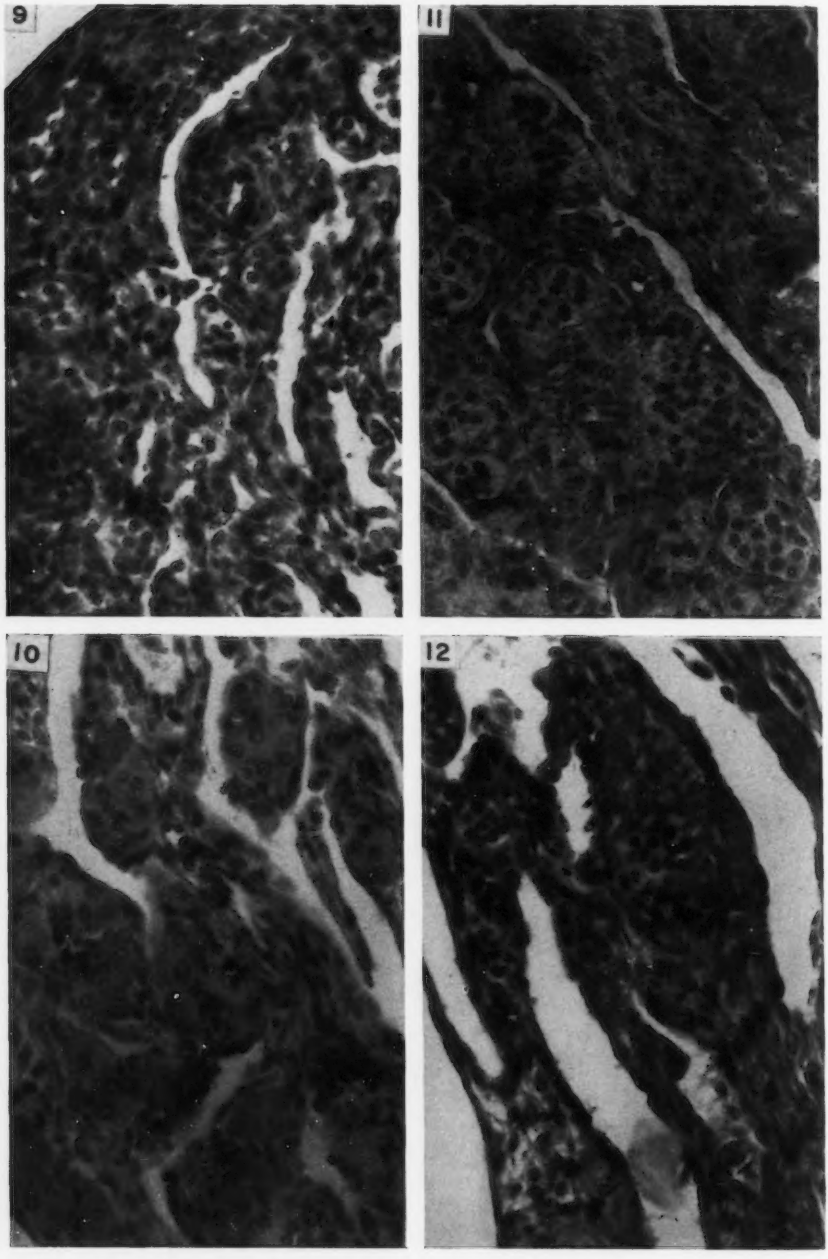


FIG. 9. Part of right gonad from 4-weeks old pullet. Note clusters of partly vacuolated medullary cord cells in connective tissue strands. ×400.

known that this powder contained high concentrations of gonadotrophic hormones, since normal female chicks treated with aliquots of the same powder showed comb-growth of the same magnitude as birds treated with 100–140 I.U. of pregnant mare serum. Also, precocious follicular growth was induced by the treatment. In addition, small amounts of adrenocorticotrophic and thyrotrophic hormones were probably present (11).

During the 5 weeks of treatment comb-sizes of these pituitary-treated poulards were the same as those of untreated poulards, indicating no stimulation of endocrine function of the rudimentary gonad. Gross and microscopic examination of the gonads revealed no stimulation of growth or differentiation of medullary tissue when compared with controls. No cortical tissue was found in the 9 gonads.

*D. Effects of Estrogen on the Development of the Right Gonad*

One hundred and fifty-four birds were sinistrally ovariectomized at three weeks of age and received, subcutaneously, a pellet of estradiol, weighing 25 mg., at the time of the operation. These birds were killed at the same intervals as the untreated poulards (Experiment B).

Effects of estrogen on the rudimentary gonad could be observed as early as one week; all or most of the medullary cord cells were vacuolated and their nuclei were smaller and denser than those of untreated poulards (compare Figs. 10 and 11). The general histological picture, however, was similar to that of the rudimentary gonad of a normal pullet of the same age (Experiment A). The gonads recovered at all subsequent intervals (2–28 weeks) were also composed of strands of connective tissue surrounding groups of vacuolated cells and could not be distinguished from gonads of estrogen-treated birds killed one week after ovariectomy (Fig. 7). Growth and differentiation of the medullary tissue of the right gonad had been completely arrested by estrogen-treatment.

No gonadal tissue could be found in 11 of the birds. Cortical tissue, containing ovarian follicles, was found in 32. The amount of cortical tissue, size of follicles, and mitotic activity of cortical cells were similar to those in the gonads of untreated poulards.

*E. Effects of Avian Gonadotrophins on Estrogen-Inhibited Right Gonads*

It seemed possible that the suppression of growth and differentiation in estrogen-treated right gonads, could be due to pituitary-inhibition. That the pituitaries of these birds had been affected by the estrogen-treatment

FIG. 10. Part of right gonad from poulard one week after ovariectomy. Note dense cytoplasm of medullary cord cells and large vesicular nuclei. ×400.

FIG. 11. Part of right gonad from estrogen-treated poulard one week after ovariectomy. Note vacuolated medullary cord cells, with dense nuclei. ×400.

FIG. 12. Part of right gonad from androgen-treated poulard one week after ovariectomy. Note small partly vacuolated medullary cord cells. ×400.

was indicated by a consistent reduction in pituitary-weight in the treated as compared with untreated poulards.

To test this hypothesis, 20 chicks, sinistrally ovariectomized at three weeks of age and implanted with estrogen pellets, were given daily (except Sunday) subcutaneous injections of the saline suspension of chicken-pituitaries described previously. The daily dosage was increased from approximately 4 mg. to 20 mg. of dry powder as the age of the birds increased. These poulards were killed 1, 2, or 8 weeks after ovariectomy.

Comb-growth of most untreated poulards (Experiment B) indicated androgen-production at 8 weeks, whereas the combs of estrogen-treated poulards, including those receiving the pituitary-powder, were small and pale.

After one week, the medullary cord cells in the right gonads of poulards treated with both estrogen and pituitary-powder were less vacuolated than were those in birds treated with estrogen alone, but not so granular as in untreated poulards. The histological appearance of the gonads was similar to that of the rudimentary gonad from a normal pullet of the same age. At 2 and 8 weeks the gonads resembled those of poulards treated with estrogen alone except for the granular appearance of the medullary cells. In one bird, killed at 8 weeks, there were small groups of well-differentiated tubules containing germinal cells. This bird differed in no other detected respect from the others; the comb-size and weights of pituitary, adrenals and thyroids were well within the range of the other poulards similarly treated.

*F. Effects of Androgen on the Development of the Right Gonad*

One hundred and forty-five birds were ovariectomized at three weeks of age and implanted subcutaneously with a pellet of testosterone, weighing approximately 38 mg., at the time of the operation. These birds were killed at the same time intervals as the untreated poulards and the estrogen-treated poulards (Experiments B and D) with the exception of the 28 weeks group.

The effects of androgen could be detected after one week of treatment and differed from the effects of estrogen; the medullary cord cells were smaller and not so vacuolated as in the estrogen-treated gonads. The amount of gonadal tissue seemed to be reduced, so that fibrous tissue predominated (Fig. 12). A small, fibrous area including only a few medullary cells was characteristic of the androgen-treated gonads of older poulards. In some of the birds killed at 8 and 10 weeks the androgen-pellets were small and almost completely absorbed. In the gonads of these birds a few mitotic figures and an increase in the size of medullary cord cells were observed. Such a gonad is shown in Figure 8.

No gonadal tissue could be found in 23 of the androgen-treated poulards. Cortical tissue was present in 15. The amount and differentiation of the cortical tissue were similar to those of gonads from untreated poulards.

The unabsorbed portions of androgen-pellets were removed from 14 birds after one or two weeks. The birds were killed 1, 2, 4, 6, 8, and 10 days later. From 1 through 8 days, the gonads showed the typical androgen-inhibition. However, ten days after the removal of the pellet a few mitotic figures were noted, the medullary cord cells had increased in size and number and the cytoplasm was more granular.

*G. Effects of Avian Gonadotrophins on Androgen-Inhibited Right Gonads*

Sixteen birds, sinistrally ovariectomized at three weeks of age and implanted with pellets of testosterone, were injected daily with the chicken-pituitary powder. These poulards were killed 1, 2, or 8 weeks later. Except for a slight increase in the number, size, and granularity of the medullary cord cells in a few cases, no effect of the pituitary powder was noted.

*H. Effects of Estrogen and Androgen on Pituitaries*

The average weights of anterior lobes from all control, estrogen-treated and androgen-treated poulards were 6.5, 4.4, and 5.0 mg., respectively. For each of these groups the linear regression of pituitary weights on age was fitted by the method of least squares. The slopes of these three lines were 0.08477 for controls, 0.03883 for estrogen-treated, and 0.07398 for androgen-treated. Since the slopes of the lines for control and androgen-treated poulards were not significantly different at the 5% level, and since the average age of the birds in these two groups was the same, the mean pituitary-weights of the two groups could be compared statistically. The reduction in weight of the pituitaries of androgen-treated birds was highly significant ($P =$ less than .0002). The slope of the estrogen-age-line was significantly less than that of the other two ($P = .003$). This indicated that the depression of pituitary weight in estrogen-treated poulards became relatively greater with increasing age. At 6, 8, and 10 weeks, the pituitaries of these birds weighed significantly less than those of androgen-treated poulards ($P = .012, .035, .002$, respectively).

Examination of serially sectioned anterior lobes revealed easily recognizable differences between the glands of control and hormone-treated poulards. In sections stained with hematoxylin and eosin basophils were smaller and their nuclei were more vesicular in the treated pituitaries. In sections stained with the periodic acid-Shiff technique less Shiff-positive material was observed in the treated glands. In these preliminary studies no consistent differences were observed between the pituitaries of androgen and estrogen-treated birds.

## DISCUSSION

Cortical tissue, varying from a few isolated follicles and cortical cords to relatively normal ovaries, was found in 27 (21.3%) of 127 poulards not treated with steroids. The incidence of this tissue was essentially the same in each age-group (1–28 weeks after ovariectomy), which suggests that the

cortical component was probably present in these gonads at the time o ovariectomy. One can conclude that the presence of cortical tissue is no rare and that one might expect to find it in approximately one-fifth of th rudimentary gonads of the light-brown Leghorn.

Since cortical tissue, similar in amount and degree of differentiation t that of untreated poulards, was found in 37 (21.3%) of 174 right gonad from estrogen-treated poulards, it can be concluded that estrogen had n effect on the incidence or differentiation of the cortical component. Th same conclusion can probably be made for androgen. Although the incidence of cortical tissue was lower in androgen-treated poulards (20 of 161 o 12.4%), the decreased incidence was not significant at the 5% leve ($\chi^2 = 3.439$).

Although both hormones inhibited the development of medullary tissue, their effects were consistently different. Estrogen-treatment seemed to arrest further growth and differentiation; androgen-treatment resulted in regression. This regression proceeded to completion in 23 birds (14.3%); no gonadal tissue could be identified in serial sections of the Wolffian body and adjacent tissue. Statistically, the complete suppression of gonadal tissue in 23 androgen-treated birds was significant when compared with the figures for control (3 or 2.4%) and estrogen-treated (11 or 6.3%) poulards (P = less than 0.01).

Kornfeld and Nalbandov (3) reported that estrogen was more effective than androgen in inhibiting the rudimentary gonad in the white Leghorn. Their experiments differed from ours in that hormone-treatment was started several weeks after ovariectomy, by which time, in the brown Leghorn, the rudimentary gonad has already begun to grow and differentiate.

Although the pituitary seems necessary for maintaining the established right gonad in a functioning state (3), its role in the initiation of development and differentiation of this gonad is not so clear. Benoit (7) suggested that the inhibition of the right gonad by the functional left one was due to lack of adequate pituitary gonadotrophins. This seems unlikely when you consider that high concentrations of exogenous gonadotrophins fail to stimulate the growth of the right gonad in intact females (12), as well as in poulards. Furthermore, Taber, Salley and Knight (13) have shown that normal differentiation of the right gonad takes place in poulards subjected to chronic inanition, a condition called "pseudo-hypophysectomy" by Mulinos and Pomeranz (14).

The experiments reported here fail to provide evidence for or against the necessity for pituitary hormones in early differentiation. However, they do show that the suppression of medullary tissue by androgen or estrogen was not due to pituitary-inhibition alone, for simultaneous treatment with chicken-pituitary powder (known to stimulate endocrine and gametogenic functions in the gonads of young chicks) failed to prevent the inhibition of medullary growth and differentiation. Furthermore, androgen caused the

greater inhibition of gonadal tissue whereas estrogen caused the greater inhibition of the pituitary.

A constant finding in the gonads of all estrogen-treated poulards was the presence of vacuolated medullary cord cells. Clusters of vacuolated cells were also observed in cortical tissue. The occurrence of similar cells in the ovaries and testes of chickens has been shown to be correlated with decreased levels of gonadotrophins and decreased secretion of androgen and estrogen. It has been demonstrated that the vacuoles in these cells contained cholesterol. On the basis of these observations it was postulated that these vacuolated cells are inactive interstitial cells (12, 15, 16). This suggests that the presence of similar vacuolated cells in the gonads of estrogen-treated poulards was due to decreased endogenous gonadotrophins resulting from the inhibition of the pituitary.

The fact that simultaneous treatment with chicken-pituitary powder caused a decrease in the vacuolization and an increase in the granularity of the cytoplasm of these cells supports this hypothesis. However, since the combs of birds treated simultaneously with estrogen and pituitary powder showed no increase in vascularity or size, the stimulus of the exogenous gonadotrophins was not sufficient to induce a detectable increase in androgen-secretion.

If one assumes that the vacuolated cells in the gonads of estrogen-treated birds contained cholesterol, the almost complete absence of such cells in androgen-treated poulards could be due either to inhibition of cholesterol formation or to the conversion of cholesterol into a secretory product. The latter alternative would require the presence of gonadotrophins, and seems unlikely when one considers the high concentration of androgen and the probable consequent suppression of pituitary secretion. The former alternative would suggest that androgen and estrogen exert qualitatively different effects on the pituitary, or that the lack of cholesterol synthesis was not a result of pituitary depression. Since simultaneous treatment with pituitary powder had little effect on the androgen-treated gonad, it seems probable that androgen interfered with cholesterol synthesis by acting directly on the medullary tissue.

A different interpretation of somewhat similar results has been given by Greep and Jones (17), who observed an increase in cholesterol-filled cells in ovaries of rats treated with estrogen, but a decrease in those treated with androgen. They postulated that both hormones were acting directly on the ovary, with estrogen aiding the formation of cholesterol and androgen depleting the cholesterol, possibly by inhibiting its formation.

In conclusion, neither estrogen nor androgen had a significant effect on the development of the cortical component of the rudimentary gonad. Both hormones inhibited the development of the medullary component but the degree of inhibition was significantly greater in androgen-treated poulards. Suppression of endogenous gonadotrophins by the sex hormones

possibly contributed to, but was not the major factor in preventing differentiation. It is probable that the inhibition of medullary tissue in the right gonad was primarily due to the direct and specific action of the two sex hormones on this gonadal tissue.

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# THE REACTION OF SEVERAL PROTEIN HORMONES WITH PERIODATE[1]

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ABSTRACT

The action of periodate on FSH, ICSH, TSH, growth hormone, HCG and PMSG has been investigated. With the exception of growth hormone, all the other hormones show markedly diminished activity after treatment with periodate. Additional information is presented on the effect of oxidation with periodate on the biological activities of α-corticotropin and lactogenic hormone. It is suggested that periodate be employed to decrease the TSH and ICSH activities in growth hormone preparations; these activities arise from contaminants in such preparations. Finally, it has been shown that periodate-treated growth hormone is still capable of partially maintaining the adrenal weights of rats that have undergone hypophysectomy.

IN PREVIOUS publications from this laboratory, the use of periodate has been reported for the determination of the N-terminal serine of α-corticotropin (1) and of the N-terminal threonine of lactogenic hormone (2). Subsequently, it was demonstrated by Dixon (3) that corticotropin, when treated with periodate, lost its adrenal-stimulating activity, although its melanocyte-stimulating activity was unaltered. Recently, Geschwind and Li (4) reported that periodate-treated lactogenic hormone was active as a crop-sac stimulator, thus proving that the α-amino group of this protein was not necessary for its activity. Thus, of the six recognized hormones of the anterior pituitary, two possessing N-terminal hydroxy-amino acids were shown to react with periodate. Of the remaining four hormones, three, (follicle-stimulating hormone (FSH), interstitial cell-stimulating hormone (ICSH), and thyroid-stimulating hormone (TSH)) are generally considered to be glycoproteins (5) and, as such, may be expected to react with periodate. Indeed, the reaction of glycoproteins with periodate is the basis of a histochemical staining procedure devised by MacManus (6), and applied to the pituitary by Pearse (7). In conjunction with other procedures, it has been used to identify those cells presumably responsible for the secretion of FSH, TSH, and ICSH.

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hormone are reported herein. In addition, the results of more limited experiments with human chorionic gonadotropin (HCG) and pregnant mare serum gonadotropin (PMSG) are recorded.

## METHODS

Two preparations of thyroid-stimulating hormone (TSH) were employed; one was obtained from the Armour Laboratories and had an activity of 1.2 U.S.P. units per mg. while the other was prepared by the method of Fels *et al.* (8), and was kindly provided by Dr. E. S. Evans. The FSH (9) and bovine growth hormone (10) preparations were prepared as previously described. The ICSH preparation was the highly purified Fraction D-3 whose isolation from sheep glands was recently reported (11). The HCG was obtained from Leo Pharmaceutical Products, and had an activity of 1000 I.U. per mg.; the PMSG was a gift from the Ayerst Laboratories, and had an activity of 2500 I.U. per mg.

The TSH was assayed primarily by the $I^{131}$ discharge method of Bates and Cornfield (12); we are indebted to Dr. Bates for carrying out all except one of these assays. In addition, the hormone was injected into male rats hypophysectomized at 28 days of age, beginning 14 days post-operatively. The hormone was given in single daily intraperitoneal injections for fourteen days. On the twelfth day of injection, the animals were fasted for 4 hours and their standard metabolic rates were determined; we are grateful to Dr. E. S. Evans for these determinations. On the fourteenth day the rats were given a tracer dose of $I^{131}$ by the intraperitoneal route; 20 hours later the animals were sacrificed, and the $I^{131}$ uptake of the excised thyroid was determined. In addition, microscopic analyses of sections of the thyroid were performed.

The growth hormone was assayed by the tibia test (13, 14) in hypophysectomized rats, and also by weight maintenance in animals hypophysectomized at 28 or 40 days of age and injected with 200 micrograms of the hormone daily for 10 days. The FSH activity was estimated by an augmentation assay (15) in 21 day-old normal female rats, who received three daily injections of the FSH preparations together with a 31 $\mu$g. total dose of HCG as synergist. Twenty-four hours after the last injection, the animals were sacrificed, and the ovaries were removed and weighed. The ICSH activity was estimated according to the increment in ventral prostate weight (16) following 4 daily subcutaneous injections given to rats hypophysectomized at 21 days of age, beginning 2 days post-operatively. The HCG activity was determined in 21 day-old normal male rats according to the increment in the weight of the ventral prostate after 3 daily intraperitoneal injections, and the PMSG was assayed in female rats by the FSH assay method, but without benefit of the synergizing dose of HCG. All rats employed in this investigation were of the Long-Evans strain.

Periodate oxidations were generally carried out in 0.25 to 1% solutions of the hormone in 0.1*M* $NaHCO_3$, to which sufficient 0.1*M* sodium metaperiodate (freshly prepared) was added to give a final concentration of the reagent of from 0.01*M* to 0.02*M*. The reaction was allowed to proceed for 5 to 10 minutes at room temperature; after that time, either excess glucose was added to the reaction mixture to reduce the excess periodate, or else the reaction mixture was immediately diluted and dialyzed in the cold. In general, little difference was observed in the results obtained by the two methods of terminating the reaction. On a number of occasions control preparations were submitted to the complete procedure outlined above, except that the periodate was either omitted or reduced with an excess of glucose before it was added to the solution of the hormone. In no instance was any loss of activity ever observed with any hormone under the control conditions.

## RESULTS

*Thyroid-stimulating hormone:* Three different samples of peridate-treated TSH (Armour) were prepared in the course of a five-month period. The three were assayed by the $I^{131}$ discharge assay method along with a control preparation whose activity was found to be approximately 1 U.S.P. unit per mg., a value that essentially confirms the estimate of activity supplied with the original preparation. After treatment with periodate, these samples possessed activities of 0.1, 0.09 and 0.15 U.S.P. units per mg., indicating losses of activity amounting to from 85 to 90%.

Subsequently, a more highly purified TSH preparation was used in an attempt to confirm these findings. Samples of two different batches of TSH prepared by the method of Fels *et al.* (8) were treated with periodate on a

TABLE 1. THE EFFECT OF PERIODATE TREATMENT ON THE BIOLOGICAL ACTIVITIES OF THYROTROPIC HORMONE

| Experimental group | Dose | Thyroid weight | Standard Metabolic rate | 20-hour thyroid $I^{131}$ uptake* |
|---|---|---|---|---|
| | μg./d | mg. | Cals./m.²/hr. | % |
| Control (5)† | — | 4.9±0.4‡ | 22.5±1.5 | 0.19±0.04 |
| TSH-untreated (5) | 100 | 7.8±0.6 | 27.6±1.9 | 3.04±0.79 |
| TSH-treated (5) | 100 | 6.1±0.7 | 21.5±0.8 | 1.29±0.17 |
| Control (6) | — | 4.8±0.2 | 21.6±0.7 | 0.11±0.02 |
| TSH-untreated (6) | 500 | 7.9±0.5 | 31.0±0.8 | 4.91±1.14 |
| TSH-treated (6) | 500 | 6.4±0.4 | 25.6±0.5 | 2.34±0.56 |

* % of injected dose.
† No. of animals per group in parentheses.
‡ Mean ± standard error.

number of occasions. These preparations were then subjected to extensive and varied assays for TSH activity; they were injected for 14 days into assay animals, and the thyroid weights and histology, the standard metabolic rates, and the 20-hour $I^{131}$ uptake were determined. The results of some of these determinations for two separately-prepared samples of periodate-treated TSH, based upon standard curves, obtained by Dr. E. Evans, for the change in metabolic rate and $I^{131}$ uptake with increasing dose of TSH, are presented in Table 1; it is estimated that 10 to 20% of the various activites still remained after treatment with periodate. Histological examination of the thyroids showed marked loss of thyroid-stimulating activity in the periodate-treated groups (Fig. 1), confirming the extensive destruction of activity found for the other parameters. Finally, one such preparation was treated with periodate and then aliquots of both the untreated and treated hormone were assayed at 4 dose levels each by Dr. F. S. Greenspan,[2] using the $I^{131}$ discharge assay of Bates and Cornfield (12). Statistical analysis revealed that after treatment with periodate, 12.3%

[2] We are indebted to Dr. Greenspan for performing these assays.

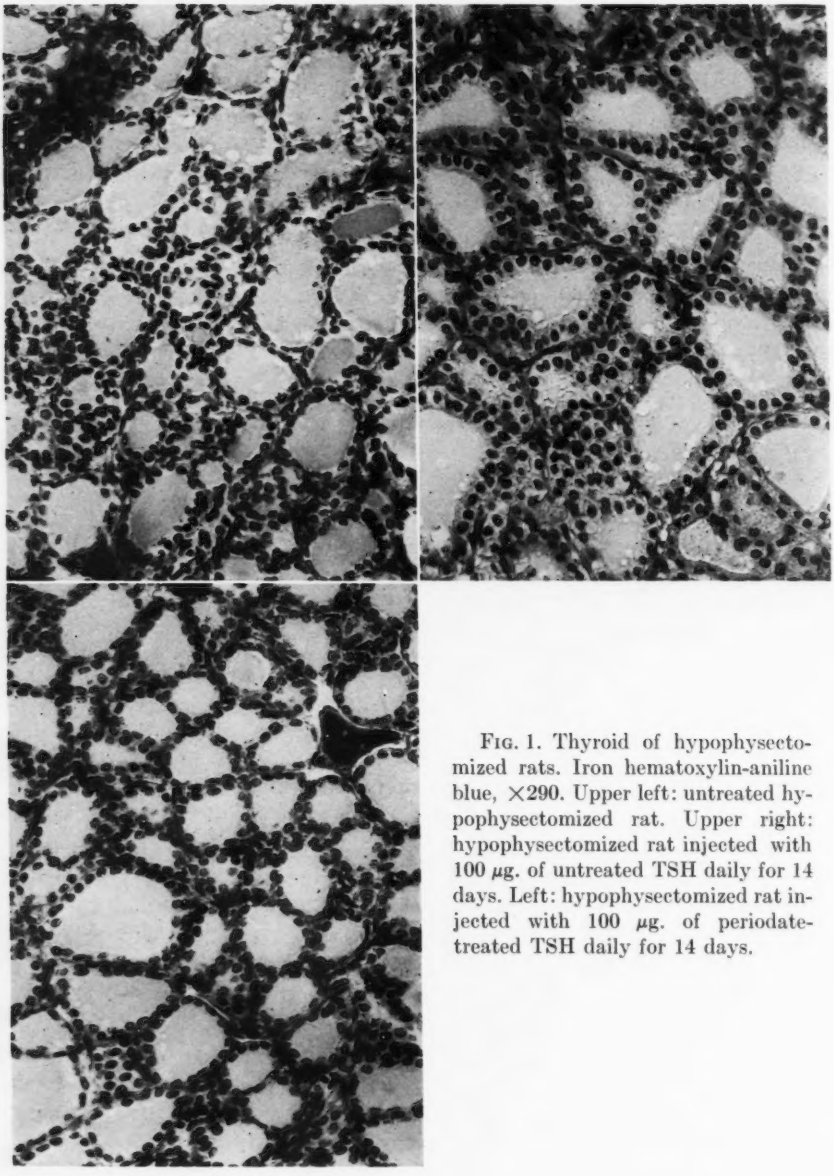

FIG. 1. Thyroid of hypophysectomized rats. Iron hematoxylin-aniline blue, ×290. Upper left: untreated hypophysectomized rat. Upper right: hypophysectomized rat injected with 100 μg. of untreated TSH daily for 14 days. Left: hypophysectomized rat injected with 100 μg. of periodate-treated TSH daily for 14 days.

(range: 5.7 to 26.3, at the 95% confidence interval) of the original activity remained.

*Growth Hormone:* Treatment with periodate of four different preparations of bovine growth hormone was completely without effect on the

TABLE 2. THE ACTION OF PERIODATE ON THE GROWTH- AND THYROID-STIMULATING ACTIVITIES OF GROWTH HORMONE PREPARATIONS

| Preparation | Before treatment | | After treatment | |
|---|---|---|---|---|
| | TSH* | Tibia tests† | TSH* | Tibia tests† |
| | U.S.P. units/mg. | Micra, aver. | U.S.P. units/mg. | Micra, aver. |
| Saline | | 165 | | |
| L2778B | 0.04 | 237 ± 3.7‡ (6)§ | 0.01 | 253 ± 3.5 (5) |
| L2769A | 0.06 | 239 ± 7.5 (5) | 0.01–0.02 | 239 ± 3.1 (4) |
| L2802 | | 236 ± 5.1 (9) | | 239 ± 4.5 (10) |
| L2722BS | | 233 ± 5.0 (9) | | 235 ± 4.0 (9) |

* Assayed by the $I^{131}$ discharge assay (12). We are indebted to Dr. R. W. Bates for these assays.
† Total dose of 40 micrograms in all growth hormone assays.
‡ Mean ± standard error.
§ Number of animals per assay in parentheses.

growth stimulating activity of these preparations, as measured by repeated spot checks, at a single dose level, by the tibia test (Table 2) and by weight maintenance experiments. The average weight increases in all groups receiving control or treated preparations was 2.5–3 gm. per day; in the uninjected controls, no change in weight was observed in the 28-day-old animals, while an average loss of 1–1.5 gm. per day was encountered for the 40-day-old animals.

In addition to the assays for growth activity, assay for TSH contamination in the growth hormone preparation was also carried out by means of the $I^{131}$ discharge method. The results of these assays (Table 2) indicate that the small amount of TSH activity in the control growth hormone preparations is markedly decreased (approximately 75%) following treatment with periodate.[3]

*Follicle-Stimulating Hormone:* Two different preparations of FSH with similar initial activity were subjected to oxidation by periodate. Assays employing the increment of ovarian weight as an index of activity indicate that in each preparation more than 85% of the activity was destroyed (Table 3), since the response to 1,000 μg of periodate-treated FSH was found to be significantly less than that to 150 μg. of the untreated preparation.

[3] Samples of the untreated and periodate-treated growth hormone preparation L 2722 BS were employed by Dr. E. S. Evans for studies on hormonal factors affecting the metabolic rates of rats. Groups of 5 or 6 rats were injected daily for 14 days, beginning at 57 days of age and 29 days after hypophysectomy, with either the control or treated preparation. On the 14th day, all animals received injections of $I^{131}$, and 20 hours later were sacrificed, their thyroids extirpated and fixed, and the $I^{131}$ uptake determined. The $I^{131}$ uptake was 0.47 ± 0.06 (S.E.) % in the uninjected controls, 1.45 ± 0.21% in those rats receiving 500 μg. of the untreated control preparation, and 0.49 ± 0.06% in those receiving an equal amount of the periodate-treated preparation. The interpretation of the histological sections of such thyroids was consistent with the $I^{131}$ findings. We are indebted to Dr. Evans for permission to cite these studies, which he will report in full detail in a separate paper.

TABLE 3. THE ACTION OF PERIODATE ON THE ACTIVITY OF THE FOLLICLE-STIMULATING HORMONE

| Preparation | Experimental group* | Total dose | No. of animals | Average ovarian weight |
|---|---|---|---|---|
| | | μg. | | mg. |
| ME-419, 423, 347B | Uninjected | — | 6 | 29.8 ± 0.9† |
| | Native hormone | 150 | 6 | 82.1 ± 8.5<br>$p \ll 0.001$‡ |
| | Periodate-treated | 150 | 6 | 34.3 ± 1.8<br>$p > 0.05$‡<br>$p \ll 0.001$§ |
| | Periodate-treated | 1000 | 6 | 46.6 ± 3.9<br>$p < 0.01$‡; $p < 0.01$§ |
| L2493BS | Uninjected | — | 6 | 29.3 ± 0.9 |
| | Native hormone | 150 | 6 | 71.4 ± 10.1<br>$p < 0.01$‡ |
| | Periodate-treated | 150 | 5 | 34.1 ± 2.4<br>$p > 0.05$‡; $p < 0.01$§ |
| | Periodate-treated | 1000 | 6 | 44.2 ± 6.6<br>$p < 0.05$‡; $p < 0.05$§ |

* All animals received a total dose of 31 μg. of HCG.
† Mean ± standard error.
‡ p value for comparison with uninjected control group.
§ p value for comparison with native hormone group.

*Interstitial-cell-Stimulating Hormone:* When two preparations of ICSH were allowed to react with periodate, a considerable loss of activity resulted, as measured by ventral prostate weight increments. Thus, whereas a total dose of 1 μg. of the starting material increased the average ventral prostate weight from 8.5 to 13.6 mg., and 2 and 10 μg. raised the weight to 16.3 and 20.1 mg., respectively, 25 μg. of the periodate-treated preparations increased the weight in 12 animals to an average of only 14.0 mg. This value is not significantly different from the results obtained with 1 and 2 μg. of the native preparation; it is significantly different ($P < 0.01$) from that obtained with 10 μg. of ICSH. Although extensive assaying was precluded by a paucity of starting material, the limited assays of the treated material at one dose level demonstrate extensive modification of activity following treatment of ICSH with periodate.

*Human Chorionic and Pregnant Mare Serum Gonadotropins:* As shown in Table 4, the activities of HCG and PMSG are found to be markedly altered following treatment with periodate. Both of these substances are known to contain carbohydrate.

## DISCUSSION

There exist numerous reports in the literature of 15 to 20 years ago on the ability of ptyalin and takadiastase to destroy the hormonal activity of

PMSG (18), HCG (19) and FSH (20). The inactivation observed was in all cases attributed to the hydrolytic effects of these crude enzyme preparations on the carbohydrate moieties of these hormones. However, ICSH activity was reported to be relatively resistant to the action of these enzymes (20), and no reports relating to the effect of carbohydrases on FSH activity have been made, so far as we are aware. It may be assumed from the information that has been supplied by the enzymic experiments, and from the successful use of periodate as a histochemical reagent, that the latter compound would be useful in promoting the destruction of hormonal activity in those glycoproteins where the carbohydrate is necessary for activity. The first such use was made by Whitten (21) who reported extensive destruction (94)% of the follicle-stimulating activity of PMSG preparations following treatment with 0.001*M* periodate for 30 minutes at pH 6.0. The interstitial-cell stimulating activity of such preparations, however, was reported to be relatively resistant to the action of periodate. These experiments have now been repeated in this laboratory (22), and considerable loss (75–80%) of both types of activity has been found. In part, these differences in results may be due to the more alkaline pH and the greater concentration of periodate used by the latter authors. Whitten subsequently reported on the effects of periodate on HCG activity (23), finding that the treated hormone showed no appreciable loss of activity (<15%) when assayed at low dose levels; however, at high dose levels the response to the treated hormone was less than the response to the untreated. Since the curves of dose versus response were no longer parallel, a

TABLE 4. MODIFICATION BY PERIODATE OF THE BIOLOGICAL ACTIVITIES OF HCG AND PMSG

| Experimental group | Male rats | | | Female rats | | |
|---|---|---|---|---|---|---|
| | Total dose | No. of animals | Average ventral prostate wt. | Total dose | No. of animals | Average ovarian wt. |
| | μg. | | mg. | μg. | | mg. |
| Uninjected controls | — | 9 | 32.5±1.7* | | | |
| HCG-untreated | 1 | 9 | 41.2±2.6 | | | |
| | 4 | 15 | 53.3±2.5 | | | |
| HCG-treated | 1 | 5 | 29.7±2.5 | | | |
| | 10 | 5 | 38.6±2.0 | | | |
| | 20 | 4 | 50.1±6.4 | | | |
| Uninjected controls | | | | — | 4 | 15.1±0.7 |
| PMSG-untreated | | | | 8 | 4 | 27.1±3.2 |
| | | | | 20 | 4 | 62.0±6.6 |
| PMSG-treated | | | | 20 | 4 | 22.6±1.9 |
| | | | | 50 | 4 | 36.4±2.4 |
| | | | | 150 | 4 | 64.6±5.2 |

* Mean ±standard error.

valid comparison of activities could not be made. The limited experience we have had with periodate-treated HCG (Table 4) indicates profound loss of activity at both low and high dose levels. Once again, the conditions for the oxidation which we employed differed from those used by Whitten, which may serve to explain the differences observed.

Subsequent to its use in connection with these two non-pituitary glycoprotein hormones, periodate was employed for the oxidation of the N-terminal hydroxy-amino acids of corticotropin (1) and lactogenic hormone (2). Data obained in this laboratory have confirmed the findings of Dixon (3) regarding the inactivation of corticotropin by periodate. For example, in one experiment, the daily injection of 15 μg. of an α-corticotropin preparation in beeswax-peanut oil for 8 days into rats hypophysectomized at 40 days of age, with injections starting on the day of operation, maintained the adrenals at 30.9 mg., whereas the weight of the glands of the control animals decreased to 12.3 mg. The injection of 200 μg. per day of a periodate-treated preparation was completely without effect, the adrenals weighing no more than those of the control animals (12.5 mg.). The weights of the thymus reflected the atrophic condition of the adrenals in the animals receiving periodate-treated corticotropin and in the control animals. In an investigation to determine whether the periodate-treated corticotropin would antagonize or inhibit the response to the native corticotropin, the two preparations were both injected at the dose levels recorded above (15 μg. untreated plus 200 μg. treated) for 8 days into 5 test animals. The adrenals of these animals weighed 28.9 mg., not significantly different from the value of 30.9 mg. found for the animals receiving the native preparation alone.

With respect to the lactogenic hormone, recent assays for luteotropic activity, as measured by deciduoma formation (24), have demonstrated that treatment of this hormone with periodate leaves the luteotropic activity unaltered. These results complement and support previous findings (4) in connection with the crop-sac stimulating activity of such preparations.

The data recorded in this paper indicate that following treatment with periodate, the biological activities of TSH, FSH and ICSH are extensively destroyed. Of some interest, however, is the finding that in the glycoprotein hormones it is virtually impossible to destroy activity completely with periodate, in contrast to the apparently complete destruction of corticotropic activity effected by this reagent. We have moderately increased the concentration of periodate and also the duration of reaction, and find that from 5 to 10% of the activity of the pituitary glycoproteins still remains, as shown by assaying such preparations at high dose levels. It would appear that such activity is inherent in the molecules whose carbohydrate components have been oxidized under the conditions herein employed. It is quite possible that oxidation carried out at a more acidic pH would result

in the oxidation of carbohydrate components resistant to oxidation in 0.1*M* $NaHCO_3$; however, the oxidation of the N-terminal hydroxy-amino acid of corticotropin would not be favored under the more acidic conditions (25).[4] Furthermore, it must be borne in mind that growth hormone is inactivated in acid solution (26).

In sum, of the six recognized hormones of the anterior pituitary, FSH, ICSH and TSH, the three glycoproteins, have their biological activities extensively modified by periodate action. This action is presumably concerned with oxidation of carbohydrates in these three instances, although it is conceivable that hitherto undemonstrated N-terminal hydroxy-amino acids, which are necessary for activity, are involved. No attempt was made to measure the extent of oxidation of the carbohydrate components since none of the glycoprotein preparations available to us can be considered chemically pure. Of the other three hormones, two, corticotropin and lactogenic hormone, react with periodate by virtue of their N-terminal amino acids. In these two, corticotropic activity is destroyed, but the lactogenic hormone, although deprived of its normal N-terminal threonine, appears fully active. One hormone alone, the growth hormone, apparently does not react at all with periodate. Thus five of the six hormones are chemically altered, and four of the six undergo considerable inactivation.

The unique stability of growth hormone to reaction with periodate is of interest and may be used to advantage. For example, it is recognized (27) that the most difficult contaminants to remove from growth hormone preparations are ICSH and TSH. The removal of the latter hormone is particularly important, for its presence may contribute to the metabolic effects attributed to growth hormone. As shown in Table 2, inactivation, amounting to approximately 75%, of the TSH contaminant in growth hormone is obtained following treatment with periodate. From the results obtained with ICSH, similar inactivation may be expected for any ICSH contaminant in growth hormone. It is therefore suggested that treatment with periodate be used as a routine procedure to minimize the TSH and ICSH contaminants in growth hormone. Thus greater biological purity

[4] Although a non-diffusible aldehyde (in the form of an α-N-glyoxylyl derivative) is formed in both corticotropin and lactogenic hormone following treatment with periodate in 0.1*M* $NaHCO_3$, the acidic conditions under which oxidation with periodate takes place in the standard histochemical procedures (e.g., unbuffered 0.5% periodic acid (6)), would not favor the *in situ* oxidation of the N-terminal hydroxy-amino acids of these hormones. For this reason, the cells secreting these hormones presumably cannot be successfully revealed by these usual histochemical procedures. In addition, the small concentration in the pituitary gland of corticotropin, if indeed this hormone is not dissolved out by the fixation procedures, and the low concentration of aldehyde in periodate-treated lactogenic hormone (one group per 25,000 molecular weight) contribute to the failure of the histochemical procedures to reveal these hormones.

will be attained, although chemical purity will not be altered. (Assuming pure TSH to have an activity of 20 units per mg., a contamination of 0.06 U per mg. represents a 0.3% contamination.)

One word of caution should be introduced regarding the "non-specific" oxidative effects of periodate seen upon prolonged reaction of proteins with this reagent. When bovine serum albumin was allowed to react for 72 hours with 0.033*M* periodate, losses of cystine, cysteine, tyrosine and tryptophan were observed (28). Ribonuclease, when submitted to 0.05*M* periodate at pH 5.1 for 3 hours, lost approximately 40% of its activity; inactivation was complete in 24 hours (29). Consequently it is suggested that the duration of the reaction with periodate be limited to intervals not much longer than those used in this paper.

Finally, the fact that corticotropic activity is destroyed by periodate whereas growth hormone activity is not, makes it possible to determine whether or not the adrenal weight-stimulating activity in growth hormone preparations is due to a contamination by corticotropin. In a typical experiment, rats were hypophysectomized at 40 days of age, and, beginning with the day of operation, were injected daily for 11 days with 200 μg. of either a control or a periodate-treated growth hormone preparation. At sacrifice on the twelfth day, the average weight of the adrenals of the uninjected animals was 9.0 mg. (range: 6.8–11.1), of the animals injected with the untreated hormone, 15.4 mg. (range: 13.0–18.6), and of the animals receiving the periodate-treated preparation, 14.6 mg. (range: 13.0–17.9). It would therefore appear that the effects of growth hormone in partially maintaining the adrenal weight are an intrinsic property of this hormone, and are not due to a corticotropin contaminant. Other evidence supporting this view is given elsewhere (30, 31).

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# THE PATHOGENESIS OF "ADRENAL REGENERATION" HYPERTENSION

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ABSTRACT

Adrenal regeneration hypertension which follows unilateral nephrectomy and adrenalectomy and contralateral adrenal enucleation of weanling rats receiving 1% sodium chloride solution in lieu of drinking water, does not occur if acute adrenal cortical insufficiency is avoided. It is concluded, therefore, that this form of hypertension is not a consequence of any abnormal activity of the regenerating adrenal but of the superimposed effect of acute cortical insufficiency. It is suggested that the condition is comparable to and probably a result of potassium depletion or possibly other electrolyte disturbances imposed on the other factors which induce the renal deficiency responsible for the development of hypertension.

THE demonstration by Skelton (1) that unilateral adrenalectomy with enucleation of the opposite adrenal gives rise to hypertension in rats sensitized by unilateral nephrectomy and 1% saline (as drinking water) has suggested that the adrenal may play an important role in the pathogenesis of hypertension. Other data indicate that although the adrenal cortex may play a permissive role in the maintenance of an elevated blood pressure in the hypertensive animal, this gland is not responsible for the development of this condition (2, 3). One might assume, of course, that the regenerating adrenal elaborates an abnormal hormone or an unphysiological ratio of normal hormones which induces the observed hypertension. On the other hand, it seems more probable that the development of hypertension is a consequence of the effect of temporary adrenal cortical insufficiency superimposed on the effects of unilateral nephrectomy and high salt intake, both of which procedures, alone, tend to elevate the blood pressure slightly. The experiments to be described were devised to further elucidate these possibilities.

## MATERIALS AND METHODS

Series of ten weanling rats of a piebald Long-Evans strain reared in the laboratory were subjected to unilateral nephrectomy and adrenalectomy with enucleation of the contralateral adrenal as described by Skelton (1) and given 1% sodium chloride solution in lieu of drinking water. Control animals were subjected to unilateral nephrectomy only without subjecting either adrenal to any surgical manipulation. Other animals were subjected to Skelton's procedure but were injected subcutaneously once daily with corti-

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sone and desoxycorticosterone in an amount previously determined to be adequate to maintain a normal growth curve of bilaterally adrenalectomized rats (*viz.* 1 mg. of each drug per rat daily) when fed their usual diet under normal laboratory conditions. In order to prevent any appreciable adrenal cortical insufficiency during the period prior to the development of sufficient regeneration of the gland to maintain normal adrenal function, the dose of hormones administered was gradually reduced initially over periods varying from a week to a month. A period of 10 days proved adequate for avoiding evidence of cortical insufficiency as reflected in the growth-curve of the animals. This period of time was, therefore, adopted during which the dose of the hormones was reduced by 0.1 mg. daily so that no substitution therapy was given after the 10th day following enucleation of the adrenal. In order to minimize the traumatic effects of the operative procedure, the animals were allowed one week to recover from the preliminary operation of unilateral nephrectomy and adrenalectomy before subjecting them to the enucleation of the remaining adrenal. After a period of 8 weeks, blood pressures were determined daily on the trained unanesthetized animals by the method of Williams, Harrison and Grollman (4).

A total of 7 dogs were also subjected to the same operative procedures and maintained by the administration intraperitoneally of a solution having the composition of extracellular fluid. This procedure permits the maintenance of animals in good condition following adrenalectomy without recourse to hormonal therapy (5). After a week of treatment, sufficient regeneration of the adrenal had occurred to permit survival without further therapy.

## RESULTS

The results of the present study are summarized in Table 1. The first experiment cited gives the control levels of blood pressure in untreated animals. The second experiment indicates that unilateral nephrectomy and the administration of sodium chloride in place of drinking water results in a slight but definite increase in blood pressure. The difference is not great but in view of a larger series of studies previously reported (6) in which

TABLE 1. THE EFFECT OF VARIOUS PROCEDURES ON THE ARTERIAL BLOOD PRESSURE OF THE RAT. VALUES ARE AVERAGES OF 6 READINGS ON SUCCESSIVE DAYS

| Operative procedure | Post-operative procedure | Number of animals | Final blood pressure (systolic) in mm. Hg. | | | |
|---|---|---|---|---|---|---|
| | | | Minimum | Maximum | Average | Variation from mean |
| None (controls) | None | 20 | 100 | 120 | 114 | ±10 |
| Right nephrectomy | 1% NaCl in place of drinking water | 12 | 114 | 130 | 120 | ± 8 |
| Right nephrectomy and adrenalectomy, with enucleation of left adrenal | 1% NaCl in place of drinking water | 12 | 140 | 170 | 156 | ±15 |
| Right nephrectomy and adrenalectomy, with enucleation of left adrenal | 1% NaCl in place of drinking water plus cortisone and desoxycorticosterone as replacement therapy | 12 | 115 | 135 | 124 | ±11 |

unilateral right nephrectomy alone was performed and in view of the demonstration of the hypertensive effect of sodium chloride (7), the observed increase is probably significant. Hence, the effect of unilateral adrenalectomy and enucleation of the opposite adrenal as reported in the third experiment cited in Table 1 must be considered as a summation of the combined effects of nephrectomy, the administration of saline and partial adrenalectomy. The last experiment of Table 1 indicates that so-called "adrenal regeneration" hypertension may be obviated by preventing the acute phase of adrenal cortical insufficiency which follows unilateral adrenalectomy and enucleation of the contralateral adrenal.

The seven dogs which were maintained following adrenalectomy by the intraperitoneal injection once daily of a solution having the composition of extracellular fluid (5) failed to develop hypertension. Their mean blood pressures when determined at monthly intervals for a period of 10 months varied from 100 to 120 mm. Hg, which is within the range of that observed in normal animals in this laboratory. Three animals which survived the enucleation without resort to peritoneal injections also showed no elevation in blood pressure and hence it must be concluded that the procedure, unlike in the rat, does not induce hypertension.

## DISCUSSION

In interpreting the significance of "adrenal regeneration hypertension" one must consider the fact that at least three factors (nephrectomy, the ingestion of saline and temporary adrenal insufficiency) are involved in addition to any effect which the regenerating adrenal may exert. Unilateral nephrectomy results in little change in blood pressure because of the compensatory hypertrophy of the other kidney. However, a slight elevation in blood pressure of statistical significance is evident following this operation alone (6). The administration of salt, as shown by Meneely *et al.* (7) also induces an elevation in blood pressure. One need postulate, therefore, only the superimposition of a third factor induced by adrenalectomy to explain the development of "adrenal regeneration" hypertension. This factor may involve(1) the production of some secretion by the regenerating adrenal, either of an abnormal product or of a normal secretion in an abnormal amount; or (2) the result of temporary adrenal insufficiency which occurs during the short period that is required until regeneration of the gland occurs. The first-mentioned possibility has been excluded by Masson *et al.* (8). The present results would indicate that it is the temporary adrenal cortical insufficiency which is the cause of the hypertension.

The mechanism whereby a period of acute adrenal insufficiency might induce hypertension probably involves the well-established electrolyte disturbance induced by this procedure (9). As shown elsewhere (10), a short period of potassium depletion which occurs in many tissues during adrenal insufficiency (9) will induce hypertension. Such depletion super-

imposed on the other hypertension-inducing procedures involved in Skelton's experiment would account for the observed rise in blood pressure.

It would appear from the available evidence that there is no need to assume any obscure function on the part of a regenerating adrenal to account for the development of hypertension in the procedure of Skelton, but that this can be readily accounted for on the basis of known actions exerting their effect on the kidney to give rise to a form of hypertension which does not differ from that induced by other procedures and which has the same pathogenesis as that of other forms of renal hypertension. Such a conclusion is supported by the more recent observation of Skelton (11), who noted that the administration of 1 mg. of corticosterone daily prevented the appearance of "adrenal regeneration" hypertension. The induction of such hypertension by larger doses (5 mg.) of this drug would be anticipated from the well-known detrimental effects of such doses on the blood vessels of the kidney. As shown by Masson *et al.* (8), the regenerated adrenal does not produce excessive amounts of corticosterone.

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# BIOLOGICAL ACTIONS OF 17α-(2-METHALLYL)-19-NORTESTOSTERONE, AN ORALLY ACTIVE PROGESTATIONAL AGENT

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ABSTRACT

17α-(2-Methallyl)-19-nortestosterone (SC-9022) is an active progestational agent when studied in a number of biological tests. In the intrauterine assay it was estimated to be about 10 times as active as progesterone (range 5–20 times). In the subcutaneous Clauberg assay it was 25 times progesterone (12–50); in the oral Clauberg it was about 10(5–20) times the standard, progesterone administered by the subcutaneous route, and 500–2000 times orally administered progesterone. It was about 10 times as active as progesterone (a) in maintaining histamine-induced rat deciduomata, and (b) in causing increases in uterine carbonic anhydrase. SC-9022 maintained pregnancy in spayed rabbits and blocked copulation-induced ovulation in rabbits. It stimulated uterine growth in immature white mice, but antagonized the action of estrone in the same test (3–5 times progesterone). It had essentially no estrogenic effects on vaginal smears of spayed rats.

THE past several years have been marked by a growing interest in the biological activity of compounds related to 19-nortestosterone. Recent reports that various 19-nortestosterone derivatives are potent oral progestational agents have focused attention on this phase of the biology of these steroids. Hertz, Tullner and Raffelt (1) reported that 17α-ethynyl-19-nortestosterone was orally active in rabbits, and Greenblatt extended the observation to women (2). Ferin (3) and Overbeek (4) showed the 17-methyl derivative to have progestational activity in women and rabbits respectively. Pincus *et al.* (5) confirmed and extended observations on 17α-ethynyl-19-nortestosterone and compared its effects with those of the Δ 5(10) isomere of the ethynyl derivative and 17-ethyl-19-nortestosterone. Edgren (6) continued the comparisons of the Δ 5(10) ethynyl compound and its conjugated isomere. A series of 19-nortestosterones was studied for progestational effects by Saunders, Colton and Drill (7) who showed that this activity was unrelated to either androgenic or anabolic potency (8). These studies suggested that one or more compounds in this series might prove of therapeutic value as an oral progestational agent.

Our continued studies on these compounds suggested that 17α-(2-methallyl)-19-nortestosterone (SC-9022)[1] was the most potent progesta-

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[1] SC-9022 was synthesized by Dr. F. B. Colton and Mr. Richard Ray of Division of Chemical Research, G. D. Searle and Co.

tional agent of the series. The present communication summarizes these studies.

## PROGESTATIONAL ACTIVITY

Two classical manifestations of progesterone in mammals were the subject of this study; first, progestational development of the endometrium, including maintenance of pregnancy, and second, progesterone-induced blockage of pituitary gonadotropin.

### *Endometrial Tests.*

The relative progestational activity of SC-9022 was determined in estrogen-primed rabbits using intrauterine, subcutaneous and oral routes of administration, while corallary studies evaluated changes effected in uterine carbonic anhydrase concentrations. The ability of SC-9022 to maintain histamine-induced deciduomata in estrogen-primed rats and pregnancy in ovariectomized rabbits was ascertained.

### *Gonadotropic Inhibition*

Inhibition of ovulation in multiparous rabbits was employed to determine the effectiveness of SC-9022 in preventing secretion of pituitary gonadotropin.

### *Endometrial Tests*

*Intrauterine, McGinty Assay* (9). Virgin female rabbits were ovariectomized and, starting sixteen days later, they were primed for 5 days with 5 μg. estrone per day. On the day following the last priming injection they were anesthetized, the uterus was exposed and the test material was injected into a one-inch segment of the uterus. A similar segment of the contralateral horn was injected with corn oil and served as a control. Three days later the rabbits were sacrificed and the uterine segments removed and prepared for histological inspection. Upon microscopic examination, the degree of glandular proliferation in the endometrium was determined and rated on a +1 to +4 scale. A +1 response represented that type of uterus that had essentially no glandular proliferation, while a +4 response was typical of one with maximally stimulated endometrial glands. In this assay, SC-9022 was between 5 and 20 times more active than progesterone; the best estimate suggested a value of 10 (Fig. 1).

*Subcutaneous, Clauberg Assay* (10). Intact, immature, female rabbits weighing about 1 kg. were primed for 6 days with 5μg. estradiol per day. Starting on the day following the last priming injection, the rabbits were injected daily for 5 days with the test compound. On the day after the last injection, the animals were sacrificed and a segment of the uterus was taken for histological examination. The uteri were graded from +1 to +4 as above. SC-9022 appeared to have between 12 and 50 times the activity of progesterone; the best estimate suggested a value of 25 (Fig. 2).

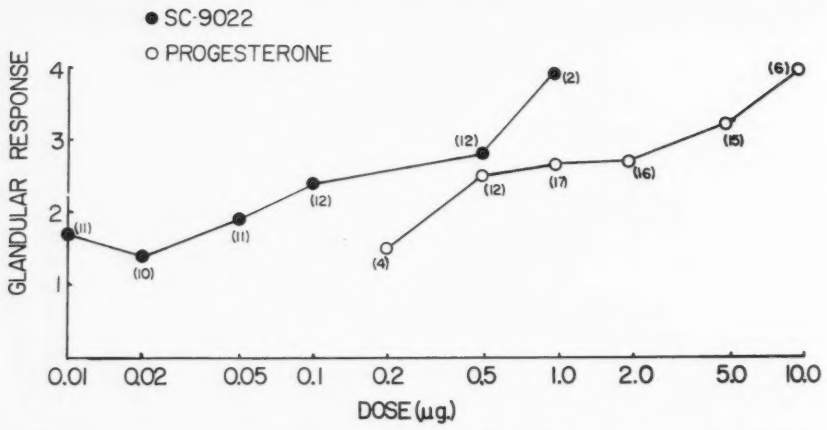


FIG. 1. Comparison of locally administered SC-9022 and progesterone upon the glandular development of the rabbit endometrium. Numbers in parentheses indicate number of animals employed at each point.

*Oral, Clauberg Assay.* Immature female rabbits were handled as above except that the test compound was administered orally. Since orally administered progesterone has only about 1% of its parenteral potency, subcutaneously administered progesterone was used as a standard for comparison in this test. Orally administered SC-9022 was found to have 5 to 20

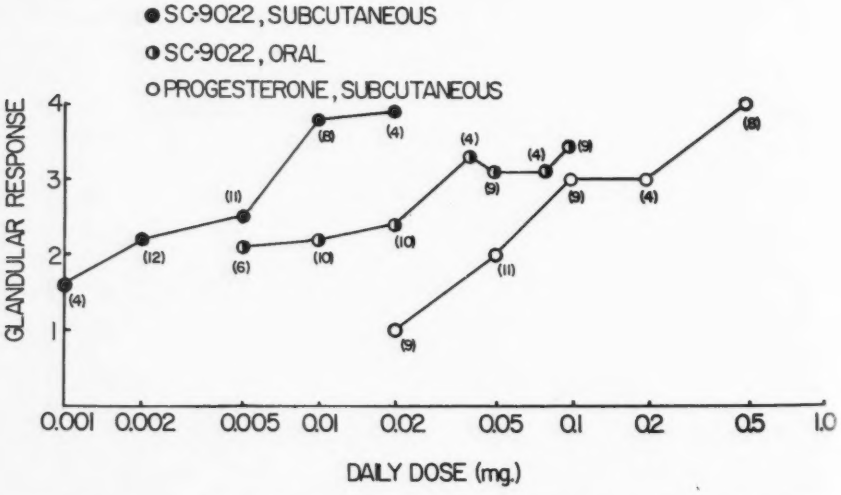


FIG. 2. Uterine endometrial response to subcutaneously administered progesterone and SC-9022 by subcutaneous and oral routes. Numbers in parentheses indicate number of animals employed at each point.

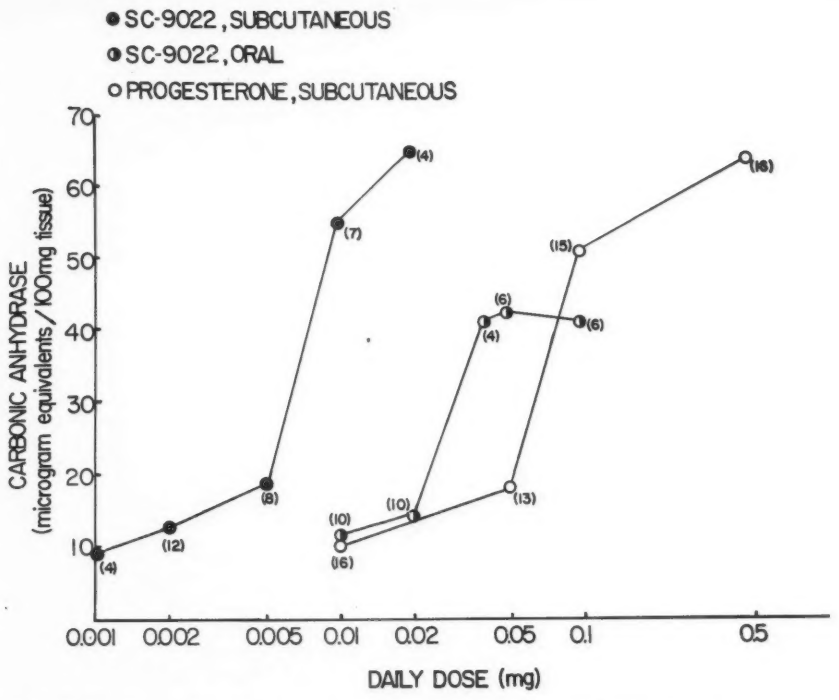


FIG. 3. Uterine carbonic anhydrase following treatment with SC-9022 and progesterone. Numbers in parentheses indicate number of animals employed at each point.

times the activity of subcutaneously administered progesterone, the best estimate was 10 (Fig. 2).

*Uterine Carbonic Anhydrase.* Uterine carbonic anhydrase has been shown to increase rapidly following the administration of progesterone to estrogen-primed rabbits (11). This increase in enzyme concentration has also been correlated with the degree of glandular proliferation in the endometrium (12). Therefore, SC-9022 was studied for its effect in inducing such changes in enzyme concentration.

Uterine segments were taken from rabbits utilized in the Clauberg assay above. The entire segment was homogenized in cold distilled water and centrifuged; the supernatant extracts were analyzed for carbonic anhydrase using the method of Philpot and Philpot (13). Enzyme concentrations were calculated by comparison with a standard curve based upon data obtained with a purified preparation (National Biochemical Corp.).

From the relative enzyme concentrations it was concluded that subcutaneously administered SC-9022 was approximately 10 times more active than progesterone (Fig. 3). SC-9022 administered orally appeared to be

about 2 to 3 times more active than the standard, progesterone by the subcutaneous route.

*Fat Deciduomata.* Female Sprague-Dawley rats weighing about 175 gm. were ovariectomized, allowed to recover for 1 week, and then given a daily subcutaneous priming dose of 1 μg. estrone for 4 days. Starting on the day following the final priming dose, the test compound was administered subcutaneously for 9 days. On the 5th day of this treatment the left horn of the uterus was exposed and 1 mg. of histamine dihydrochloride was injected into the lumen of the horn. The animals were sacrificed on the day following the

TABLE 1. COMPARATIVE DECIDUAL RESPONSES TO SUBCUTANEOUSLY ADMINISTERED PROGESTERONE AND SC-9022 IN OVARIECTOMIZED, ESTROGEN-PRIMED RATS

| Compound | Daily dose, mg. | No. animals | Mean uterine weight | | Per cent increase |
|---|---|---|---|---|---|
| | | | Right horn, mg. | Left horn, mg. | |
| Oil | — | 10 | 41.8 | 42.8 | 2.8 |
| Progesterone | 0.5 | 9 | 45.0 | 60.5 | 34.5 |
| | 1.0 | 10 | 68.6 | 113.7 | 76.9 |
| | 2.0 | 9 | 57.7 | 186.2 | 210.1 |
| SC-9022 | 0.05 | 10 | 49.7 | 91.7 | 84.5 |
| | 0.1 | 9 | 66.7 | 184.1 | 220.4* |
| | 0.5 | 9 | 86.2 | 831.5 | 883.1** |

* Significantly different from progesterone (1.0 mg.), $P < 0.05$.
** Significantly different from progesterone (2.0 mg.), $P < 0.01$.
(Wilcoxon Rank Sum Test).

final treatment with the test substance. The uteri were dissected out and the right and left horns weighed separately. By this method progesterone induces decidual growth that is related to the dose administered (14).

SC-9022 had marked deciduomatogenic activity (Table 1) and these data suggested that it was about 10 times more potent than progesterone.

*Maintenace of Pregnancy.* Limited data indicated that SC-9022 maintained pregnancy in ovariectomized-pregnant rabbits. In a series of rabbits, spayed on the 10th day of pregnancy, adequate daily doses of SC-9022 maintained the animals throughout the duration of treatment (Table 2).

### *Gonadotropin Inhibition*

When given to multiparous rabbits 24 hours prior to mating, high dose levels of this compound inhibited ovulation. Both SC-9022 and progesterone decreased the number of females that accepted males; hence the number of animals available for study of ovulation-inhibition was too small to allow valid quantitative comparisons between the two compounds (Table 3).

TABLE 2. MAINTENANCE OF PREGNANCY IN RABBITS SPAYED ON THE 10TH DAY OF PREGNANCY AND TREATED DAILY WITH SC-9022 OR PROGESTERONE. LAPAROTOMY PERFORMED AT 20 DAYS AND AUTOPSY AT 31

| Dose (mg.) | No. of rabbits | Rabbits carrying to term | Average No. of embryos at 10 days | Condition at 20 days | Average No. of fetuses at term (31 days) |
|---|---|---|---|---|---|
| Oil | 3 | 0 | 11 | resorbed | 0 |
| SC-9022 | | | | | |
| 0.05 | 4 | 0 | 10 | extensive resorption | 0 |
| 0.1 | 4 | 2 | 7 | some resorption | 3 |
| 0.5 | 8 | 7 | 9 | normal | 6 |
| Progesterone | | | | | |
| 0.5 | 3 | 0 | 7 | extensive resorption | 0 |
| 1.0 | 4 | 1 | 10 | one normal fetus | 1 |

## OTHER ENDOCRINE STUDIES

*Rat Vaginal Smear.* SC-9022 was studied for its effects in producing changes in vaginal cytology in spayed rats. In such a test estrogens generally produce cornification of the cells of the vaginal epithelium. SC-9022, on the other hand, gave a cellular response roughly comparable to that seen during proestrus in normally cycling animals; i.e., the epithelial cells were only partially keratinized.

Following the method of Biggers and Claringbold, where the absence of vaginal leukocytes is considered as a positive response (our use of this method was described in detail in a previous paper, 15), SC-9022 was estimated to have an $ED_{50}$ of 2000 $\mu$g. per rat. Estrone had an $ED_{50}$ of approximately 2.33 $\mu$g. The slope of the dose-response curve for SC-9022 was markedly more shallow than that for estrone.

In an attempt to preclude the possibility that this response was estrogenic, but with time relationships that differed from typical estrogens,

TABLE 3. INHIBITION OF COPULATION-INDUCED PITUITARY GONADOTROPIN RELEASE IN MULTIPAROUS RABBITS 24 HOURS FOLLOWING A SINGLE INJECTION OF SC-9022 OR PROGESTERONE

| Compound | Dose (mg./rabbit) | No. treated | No. accepting male | No. ovulating after copulation | Per cent failing to ovulate |
|---|---|---|---|---|---|
| Oil | — | 12 | 12 | 12 | 0 |
| Progesterone | 0.5 | 6 | 4 | 2 | 50 |
| | 1.0 | 9 | 4 | 0 | 100 |
| SC-9022 | 0.01 | 6 | 5 | 5 | 0 |
| | 0.05 | 6 | 5 | 4 | 20 |
| | 0.1 | 13 | 4 | 1 | 75 |
| | 0.5 | 13 | 7 | 1 | 86 |
| | 1.0 | 10 | 2 | 0 | 100 |

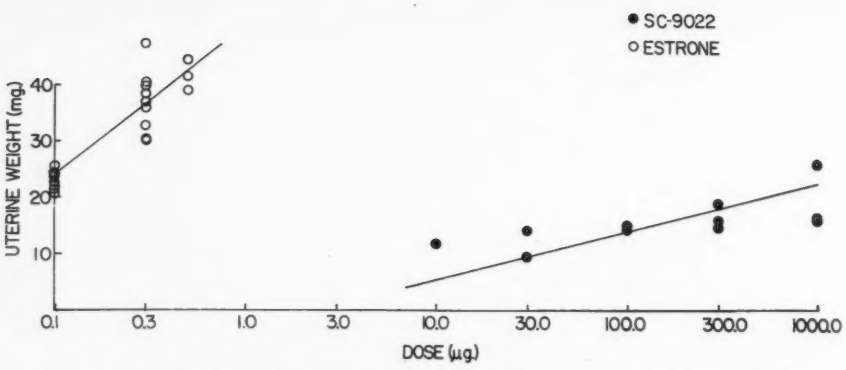


FIG. 4. Uterine growth stimulating effect of SC-9022 and estrone. Formulae for the regression lines were as follows:

SC-9022: uterine weight = $-3.12+8.48$ log dose.
Estrone: uterine weight = $-0.44+24.95$ log $100 \times$ dose
Each point represents the mean of 8 to 10 mice.

the following study was carried out: 9 spayed rats from our colony received a total of 1000 µg. of SC-9022 in two injections at 0 and 24 hours; vaginal smears were taken at 48, 56, 72, 80, 96, and 104 hours. The fresh smears were all evaluated immediately and those for 56 and 80 hours were stained with Shorr's single differential stain. All of the smears were typical of the castrate condition, with the exception of one rat that showed a "proestrus" smear at 72 hours. The stained slides simply confirmed data from the fresh readings. There was no indication of any estrogenic activity in this study.

*Metroptropic Activity.* Uterine growth in intact, immature mice can be stimulated by a variety of steroids (16). In general estrogens produce dose-response curves that have steep slopes compared with those obtained with progestins. With SC-9022, the slope of the dose-response curve was shallow and thus similar to that of progesterone (Fig. 4).

*Anti-Estrogenic Activity.* Antagonism of estrogens is a property of progestins. SC-9022 was tested as an inhibitor of estrone-induced uterine growth in mice following the method of Edgren and Calhoun (17). SC-9022 appeared to be 3 to 5 times more active than progesterone in this test (Fig. 5).

## COMMENTS

17α-(2-Methallyl)-19-nortestosterone (SC-9022) proved to be a highly active progestational agent in a series of biological tests. It shared, in common with progesterone, the following properties: effects on the endometrium of the uterus when administered by intra-uterine, subcutaneous or oral routes; ability to increase uterine carbonic anhydrase; ability to

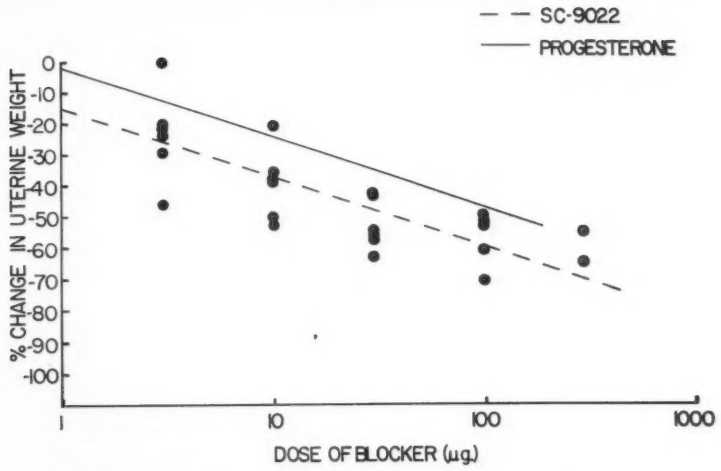


FIG. 5. The effect of SC-9022 on estrone-induced uterine growth in intact, immature mice. All animals received 0.3 µg. of estrone combined with various amounts of SC-9022. The response to estrone is represented by 0%, while a −100% response is equivalent to that obtained in oil-injected controls. Each point represents the mean of 9 or 10 mice. Curve for progesterone from Edgren and Calhoun (17). Formulae for these regression lines were as follows:

Progesterone: uterine weight = −2.28−22.20 log dose.
SC-9022: uterine weight = −15.07−22.20 log dose.

maintain deciduomata in the estrone-primed, histamine-treated uteri of spayed rats; and ability to maintain pregnancy in spayed rabbits. SC-9022 blocked copulation-induced gonadotropin release in rabbits and had metroptropic and anti-estrogenic activites.

Thus it appears that SC-9022 is a typical progestational agent; however, in contradistinction to progesterone, when administered orally, it was some 500 to 2000 times more active than progesterone by the oral route and 5 to 20 times more active than subcutaneously administered progesterone.

*Acknowledgments*

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# MECHANISM OF ANTITHYROID EFFECTS OF A SULFONYLUREA IN THE RAT[1]

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ABSTRACT

In studies designed to elucidate the mechanism of antithyroid effects of carbutamide, an hypoglycemic sulfonylurea, rats were fed a synthetic low iodine diet with carbutamide, sulfaguanidine, propylthiouracil and iodide added in various concentrations.

Carbutamide (0.125 to 1.0%) reduced $I^{131}$ uptake, increased thyroid weight and depleted glandular iodine. It was found to be 13 times as potent as sulfaguanidine and 1/200th of propylthiouracil. In contrast to sulfaguanidine and like propylthiouracil the goiters produced by carbutamide were inhibited by excess iodide. In rats fed carbutamide, $I^{131}$ accumulated in the thyroid but was promptly discharged by thiocyanate. Except for a delay of 3 days in maximum activity the antithyroid effects of carbutamide were indistinguishable from propylthiouracil.

THE sulfonylureas, a group of compounds used clinically in the treatment of diabetes millitus, have antithyroid activity. Tolbutamide, 1-butyl-3-p-tolylsulfonylurea ("Orinase"), and carbutamide, 1-butyl-3-sulfanilylurea (BZ-55), have approximately equal hypoglycemic properties, but the former compound which lacks a para-amino group has much less antithyroid activity (1, 2). This is of particular interest in view of the observation by MacKenzie in 1943 (3) that removal of the para-amino group from sulfamethyldiazine (sulfamerazine) abolished the goiter producing properties of the compound. He also observed that goiters produced in rats by sulfaguanidine and other sulfonamides were potentiated by the administration of excess iodide in contrast to inhibition by iodide of the goiters from thiouracil and other thiocarbonamides. Franklin and Chaikoff (4) using *in vitro* methods and $I^{131}$ found that the sulfonamides prevented conversion of iodine to diiodotyrosine and thyroxin but did not prevent uptake of iodide by thyroid slices. Astwood and Bissell (5) corroborated these findings in the intact rat and found no difference between thiouracil and sulfadiazine in the rate of goiter production or loss of iodine from the thyroid.

The present study was undertaken to determine if the sulfonylureas

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showed properties similar to other sulfonamides in the mechanism of goiter formation in the rat. Accordingly, the response of the thyroid gland to propylthiouracil, sulfaguanidine and carbutamide was studied with varying dietary iodine intake.

## MATERIALS AND METHODS

Immature albino rats from our breeding colony were changed from purina chow to the synthetic low iodine diet of VanderLaan and Kaplan (6), except that cod liver oil was omitted. Chemical agents were ground in a mortar with a small quantity of the diet and then mechanically mixed into the total diet. Sodium iodide was made up in 95% ethanol, spread on casein to dry at room temperature, ground in a mortar and then mixed into the remainder of the diet. For thyroidal uptake of radioiodine approximately 4 $\mu$c. of $I^{131}$ were injected intraperitoneally. In the intact rat the uptake was measured without anesthetic and was calculated by the method of Wolff (7). Thyroid glands were removed and counted under similar geometric relationships using a scintillation counter.

The iodine content of the pooled thyroids from each group (5–6 glands) was determined using the alkaline ash method of Barker.

## RESULTS

### *Experiments 1 and 2: Effects on Thyroid Weight and Iodine Content*

In the first experiment the effects on thyroid weight and total iodine content of various amounts of sulfaguanidine, carbutamide and propylthiouracil added to the diet for 13 days were compared (Table 1). The thyroid weight and iodine content of the rats on all 3 dose levels of sulfaguanidine were significantly different from the controls; the greatest effect was produced by a 2.0% concentration. Addition of 0.25 mg. of iodine per day produced no significant change in thyroid weight.

All concentrations of carbutamide produced goiters and reduced the io-

TABLE 1. EFFECTS ON THYROID WEIGHT AND TOTAL IODINE CONTENT OF SUBSTANCES ADDED TO THE LOW IODINE DIET FOR 8 DAYS. COMPARISON IS MADE WITH RATS ON THE SAME DIETS WITH ADDED IODINE

| Treatment | No iodine added | | | Iodine, 0.25 mg. daily | |
|---|---|---|---|---|---|
| | Number of rats and sex | Total iodine, mg./100 gm. thyroid | Thyroid wt., mg./100 gm. rat | Number of rats | Thyroid wt., mg./100 gm. rat |
| Sulfaguanidine 0.5% | 5 F | 12.28 | 8.31 ± 0.22* | 6 F | 7.63 ± 0.62 |
| Sulfaguanidine 1.0% | 5 F | 8.24 | 8.78 ± 0.37 | 6 F | 8.89 ± 1.53 |
| Sulfaguanidine 2.0% | 6 F | 3.10 | 12.63 ± 1.19 | 5 F | 13.84 ± 1.97 |
| Carbutamide 0.125% | 4 F | 6.21 | 10.34 ± 0.90 | 6 F | 7.74 ± 1.00 |
| Carbutamide 0.250% | 6 M | 0.67 | 18.99 ± 1.74 | 6 M | 10.32 ± 0.34 |
| Carbutamide 0.5% | 6 F | 0.24 | 25.54 ± 2.31 | 6 F | 17.24 ± 1.96 |
| Propylthiouracil 0.01% | 6 M | 0.12 | 32.61 ± 3.05 | 6 M | 12.94 ± 0.56 |
| Propylthiouracil 0.03% | 6 M | 0.086 | 30.88 ± 2.07 | 6 M | 18.40 ± 1.50 |
| Control | 9 F<br>7 M | 24.05<br>14.80 | 7.25 ± 0.32<br>7.23 ± 0.47 | | |

* Standard error of the mean.

TABLE 2. EFFECTS ON THYROID WEIGHT AND TOTAL IODINE CONTENT OF SUBSTANCES ADDED TO THE LOW IODINE DIET FOR 8 DAYS. COMPARISON IS MADE WITH RATS ON THE SAME DIET WITH ADDED IODINE

| Treatment | No iodine added | | Iodine, 0.25 mg. daily | Iodine, 5 mg. daily |
|---|---|---|---|---|
| | Total iodine, mg./100 gm. thyroid | Thyroid wt., mg./100 gm. rat | Thyroid wt., mg./100 gm. rat | Thyroid wt., mg./100 gm. rat |
| Sulfaguanidine 2.0% | 5.47 | 9.95 ± 0.18* | 15.21 ± 0.96 | 16.59 ± 3.23 |
| Carbutamide 0.15% | 5.53 | 11.12 ± 0.41 | 5.68 ± 0.11 | 7.12 ± 0.42 |
| Carbutamide 0.25% | 2.07 | 14.22 ± 3.16 | 7.17 ± 0.65 | 9.77 ± 2.49 |
| Carbutamide 1.0% | 0.31 | 25.93 ± 2.50 | 24.40 ± 2.15 | 20.03 ± 1.70 |
| Propylthiouracil 0.005% | 0.17 | 28.15 ± 2.16 | 15.21 ± 0.96 | 17.43 ± 1.03 |
| Control | 18.40 | 5.80 ± 0.07 | 6.34 ± 0.30 | 6.30 ± 0.23 |

* Standard error of the mean.

dine content of the thyroids. The antithyroid activity of 0.125% carbutamide was equal to between 1.0 and 2.0% sulfaguanidine but 0.5% carbutamide was less potent than 0.01% propylthiouracil. In contrast to the findings with sulfaguanidine, addition of iodine significantly reduced thyroid weight in all three carbutamide groups as well as in the two propylthiouracil groups.

In the second experiment the same conditions were used but changes were made in the substances added to the diet. Each value (Table 2) is the mean of 6 male rats. The goiters produced by 2.0% sulfaguanidine were significantly increased by the addition of approximately 0.25 mg. iodine to the daily ration but there was no additional response from 5 mg. daily. Addition of carbutamide to the diet again resulted in goiter production at all dose levels. The effectiveness of iodine in inhibiting goiter formation was less in the group given 1.0% carbutamide than in the other groups, but analysis of variance showed that the difference between all the groups given carbutamide alone and those given 0.25 mg. of iodine in addition was significant at the 0.001 level. The antithyroid potency of 2.0% sulfaguanidine was about equal to 0.15% carbutamide, while 1.0% carbutamide was almost equal to 0.005% propylthiouracil.

*Experiments 3 and 4. Time Characteristics of Antithyroid Effects*

In the third experiment male litter mates were distributed into 3 groups; carbutamide 0.15% was added to the diet of one group, sulfaguanidine 2.0% to another and the third group was the control. The animals were fasted the night before they were placed on the diets. The groups killed on day one were injected with $I^{131}$ approximately 8 hours after starting on the diet; twenty hours after $I^{131}$ the animals were killed and the thyroids removed, weighed, counted, and pooled for iodine determination. Subsequent

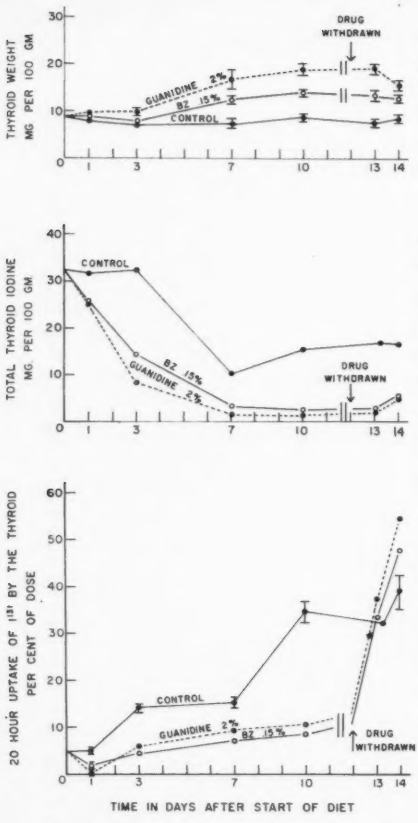


FIG. 1. Effects of low iodine diet alone and of the same diet with addition of 2% sulfaguanidine or 0.15% carbutamide. Six rats from each group were killed at intervals and measurements made of thyroid weight, total iodine content and uptake of $I^{131}$.

groups were sacrificed and counted on days 3, 7, and 10. On the 12th day all remaining animals were placed on the synthetic diet without antithyroid substances; one group was killed and counted 1 day after the antithyroid compound was removed from the diet and the last group on the following day.

The weight of the thyroid glands in the control group (Fig. 1) did not change in the 14 days on the low iodine diet despite a decrease in iodine content to one-half the starting value accompanied by a rise in uptake of $I^{131}$ from 5.0% to 35–40%. Sulfaguanidine resulted in a more rapid antithyroid effect than carbutamide so that on day one there was a significant difference in $I^{131}$ uptake between the two groups. By day three the sulfaguanidine-treated group showed greater depletion of thyroid iodine than the carbutamide group. On the same day thyroid weight in the sulfaguanidine group had increased significantly while there was still no change in the mean thyroid weight of the rats given carbutamide. From day seven to the end of the study there was no difference between the 2 groups in uptake or iodine content but goiter production in the carbutamide groups never overtook the sulfaguanidine treated animals. When the antithyroid drugs were withdrawn there was a rapid rise in $I^{131}$ uptake.

The purpose of the 4th experiment was to determine the time of onset of the antithyroid action of carbutamide as compared with that of propylthiouracil. Carbutamide (100 mg. per kg.) or propylthiouracil (5 mg. per rat) was injected intraperitoneally and the rats were then started immediately on diet containing 1.0% carbutamide or 0.01% propylthiouracil, respectively. The block in uptake of radioiodine produced by carbutamide was not complete even at day 3, while the effect of propylthiouracil was immediately maximal (Fig. 2). There was no change in thyroid weight

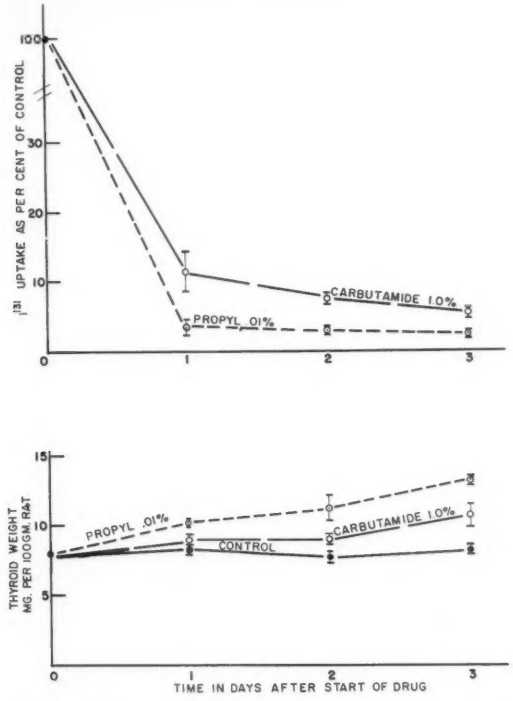


FIG. 2. Intraperitoneal injection of carbutamide (100 mg. per kg.) or propylthiouracil (5 mg. per rat) was followed by dietary administration of carbutamide (1.0%) and propylthiouracil (0.01%). Uptake of $I^{131}$ is given as per cent of control value.

from carbutamide until the 3rd day of administration, whereas a significant increase in thyroid weight was produced by propylthiouracil at the end of the first day.

*Experiment 5. Iodide Space and Response to Thiocyanate*

For this study of iodide space and response to thiocyanate rats were fed the synthetic diet for 8 days with addition of 1.0% carbutamide to the diet of one group and 0.01% propylthiouracil to another group. From each group 5 rats were selected, injected with $I^{131}$ and thyroidal uptakes measured by counting over the neck at intervals for 24 hours (Fig. 3). The pattern of uptake and loss of iodide in both treated groups was characteristic of accumulation of iodide with a block in hormone formation. The slightly greater uptake in the propylthiouracil group was probably related to the presence of larger goiters ($30.2 \pm 3.1$ mg. per 100 gms.) as compared to those in the carbutamide group ($22.1 \pm 0.6$).

Injection of $I^{131}$ into 5 additional rats from each group was followed by counts made over the thyroid at 15 minute intervals for 45 minutes. After

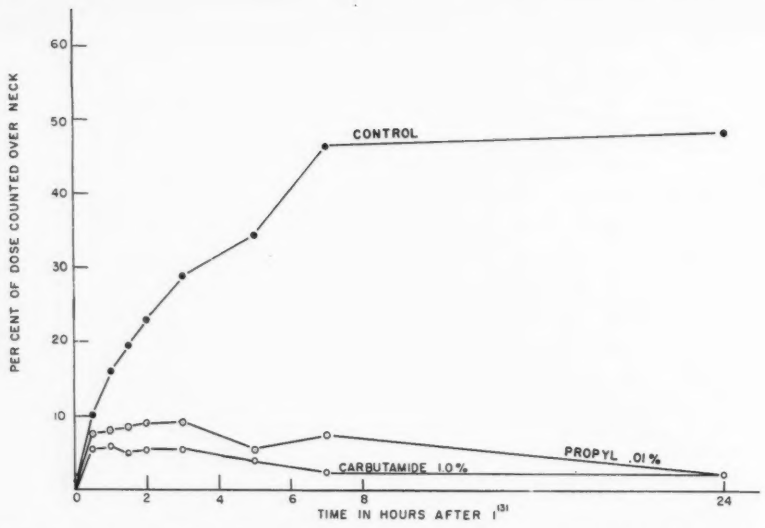


FIG. 3. Radioiodine was injected into 5 rats in each of 3 groups fed a low iodine diet for 8 days with carbutamide (1.0%) added to the diet of one group and propylthiouracil (0.01%) to another. Counts over the neck were corrected for background by subtracting one-half the count over the upper abdomen.

intraperitoneal injection of 15 mg. of potassium thiocyanate, 3 more counts at 15 minute intervals were made over the thyroid. The results (Fig. 4) again reveal that the iodide space in the propylthiouracil treated group was larger than that of the carbutamide group. Following thiocyanate administration there was a prompt discharge of $I^{131}$ from the thyroid glands of both groups, whereas the controls showed no discharge of $I^{131}$.

## DISCUSSION

Carbutamide proved in this study to be a potent antithyroid agent with effects on thyroid weight, radioiodine uptake and iodine content indistinguishable from those of propylthiouracil, except for a delay of 2–3 days before full activity was attained. Previous studies in man (1) had indicated that propylthiouracil was approximately 15 times as potent as carbutamide. In contrast, the ratio of potency in the rat in this study was approximately 200/1. This is in keeping with the previous demonstration of the extreme sensitivity of the rat thyroid to propylthiouracil (8). On the other hand, carbutamide was approximately 13 times as potent as sulfaguanidine in the present studies in the rat.

The difference between the antithyroid effects of the thiocarbonamides and sulfaguanidine is confirmed in this study; excess iodide inhibited the goiter produced by feeding propylthiouracil and increased the goiter from sulfaguanidine. In contrast to the other sulfonamides the goiter produced

by the administration of carbutamide resembles that of propylthiouracil in that it is inhibited by excess iodine. This effect was most prominent in the presence of incompletely blocking doses of carbutamide.

Histochemical techniques have provided other evidence for differentiation between the effects of thiocarbonamides and sulfonamides on the thyroid. De Robertis and Grasso (9) confirmed Dempsey's observation (10) that administration of thiourea prevents demonstration of granules in the thyroid cell typical of a peroxidase and also found that the oxidation-reduction potential of the cell was lowered. In contrast sulfonamides did not affect the oxidation-reduction potential and increased the histochemical demonstration of a peroxidase.

The mechanism whereby excess iodide inhibits goiter formation when propylthiouracil is given to rats remains obscure. It is apparent that iodine enters the thyroid but remains mainly or entirely in inorganic form (11). Iodine deficiency within the thyroid *per se* is a stimulus to hyperplasia (12) and to increased iodide accumulation by the thyroid cell (6). When iodine deficiency is combined with increased thyrotropin stimulation, both of which follow upon antithyroid drug administration, maximal stimulation

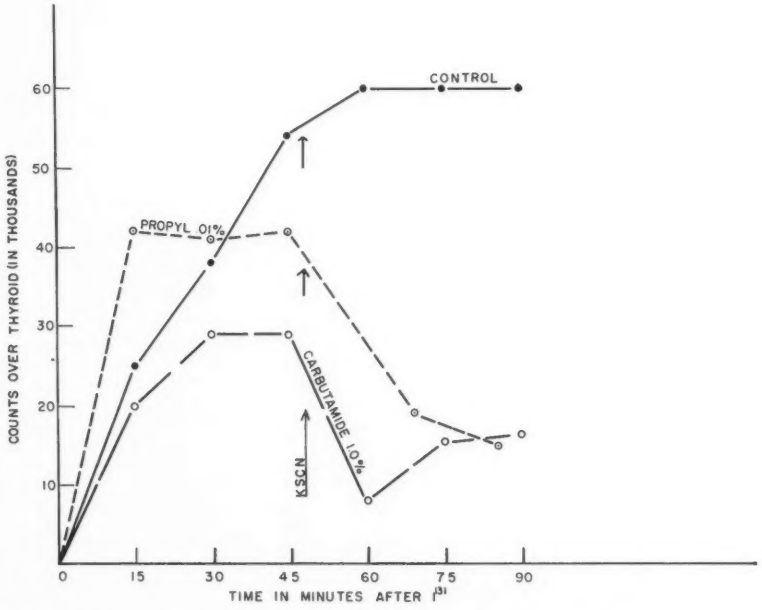


FIG. 4. Radioiodine was injected into 5 rats in each of 3 groups fed a low iodine diet for 8 days with carbutamide (1.0%) added to the diet of one group and propylthiouracil (0.01%) to another. Counts over the neck were corrected for background by subtracting one-half the count over the upper abdomen. After 3 counts at 15 minute intervals 15 mg. of KSCN were injected into all rats and 3 additional counts were made.

of the thyroid results. It is reasonable to conclude that administration of excess iodide, by increasing the iodide content of the thyroid, might remove one factor in the stimulus to the gland. Why this should not also be true in the face of sulfonamide administration is difficult to explain since iodide accumulation is not inhibited by sulfonamides (13) although organic binding is blocked. It is of interest that administration of iodide to sulfaguanidine-treated rats does not inhibit the conversion of mono- and diiodotyrosine to thyroxin and seems to accelerate this conversion in rats given propylthiouracil (14). One might suggest that a small amount of iodide escapes the block produced by the thiocarbonamide, is pushed along by excess iodide to thyroxin formation and partially inhibits thyrotropin production by the pituitary.

*Acknowledgment*

The technical assistance of Jane Paglia and Ruth L. Greene is gratefully acknowledged.

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# EFFECTS OF FEEDING THIOURACIL AND/OR THYROID POWDER UPON PUBIC SYMPHYSEAL SEPARATION IN FEMALE MICE[1]

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ABSTRACT

Thiouracil or thyroid powder added to a Purina fox chow diet markedly reduced the extent of pubic symphyseal separation in the 19-day pregnant mouse. Similarly, in nulliparous mice, thiouracil, administered for a period of 44 days, inhibited the symphyseal response to exogenous relaxin in direct proportion to the dose. Thyroid powder, on the contrary, enhanced elongation of the interpubic ligament in the nongravid mouse.

The data indicate that the influences of thiouracil upon interpubic ligament elongation are not direct drug effects, but rather appear to result from altered thyroid gland activity. The data further suggest that, although ovarian hormonal inbalances might be created by abnormal thyroid secretion, the effects of altered thyroid gland activity upon pubic symphyseal responses to relaxin are not mediated by the ovary, but rather appear to be either direct influences of the thyroid gland or reflections of the general physical condition of the animal.

THE changes which take place in the symphysis pubis of the mouse during pregnancy appear to be dependent upon precise hormonal interrelationships. Hall (1) has demonstrated the necessity of proper estrogen to progesterone ratios for a maximal symphyseal response to relaxin. More recently, Steinetz *et al.* (2) have indicated that pelvic changes in preparation for parturition in the mouse are, in addition, dependent upon a delicate balance of relaxin and progesterone.

Histologically, these changes are the resorption of the antero-medial walls of the innominate bones with transformation of the symphyseal cartilage to form, ultimately, a collagenous ligament bridging the interpubic gap (3). It has been suggested that an important factor in initiating the separation and actual relaxation is depolymerization of glycoproteins in the symphyseal connective tissues (4, 5).

It seemed quite probable that any agent capable of altering the optimal hormonal balance of estrogen, progesterone and relaxin or of interfering with any phase of the structural changes occurring in the symphysis might similarly influence the degree of symphyseal separation. The pres-

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ent study concerns symphyseal separation in pregnant and virgin female mice fed diets containing thiouracil and/or thyroid powder.

## MATERIALS AND METHODS

### *Pregnant Mice*

Albino mice (Albany strain) were bred at 110–130 days of age. Large numbers of virgin females were allowed to mate for a single night (16 hrs.), then isolated from the males, and maintained on Purina fox chow meal and tap water, *ad libitum*, for a period of 12 days. At this time those mice which were obviously pregnant were withdrawn and placed into one of four groups. Group I served as *Controls* and was further maintained on fox chow; Groups II, III and IV were fed 0.5% thiouracil, 1.0% thiouracil and 0.2% thyroid powder, respectively, in fox chow from the 12th through the 19th day of gestation. All animals were killed on the 19th day of gestation and interpubic ligaments were measured directly with vernier calipers following exposure of the symphysis pubis by dissection.

### *Virgin Mice*

A total of 515 female mice of the Albany strain, 110–130 days of age, were used in this phase of the study. Purina fox chow meal was the basic ration to which various percentages of thiouracil and/or thyroid powder[2] were added.

Group V served as the *Control* group consisting of three subdivisions: (1) intact; (2) hemi-ovariectomized; (3) ovariectomized, all maintained on fox chow without supplement. Groups VI, VII, VIII and IX, fed fox chow containing 0.5% thiouracil, 1.0% thiouracil, 0.2% thyroid and 1.0% thyroid, respectively, and Group X, fed fox chow supplemented with 0.2% thyroid powder and 0.5% thiouracil, were each subdivided similarly into intact, hemi-ovariectomized and ovariectomized subgroups. Groups XI and XII received only fox chow, but were either unilaterally (XI) or bilaterally (XII) thyroidectomized. The reproductive systems of the latter two groups were intact. All surgical procedures, i.e., unilateral (left side) or bilateral ovariectomy and thyroidectomy, were carried out 10 days prior to the start of the experiment.

All mice were maintained on their respective diets and tap water, *ad libitum*, for a period of 44 days. Each animal, including controls, received, subcutaneously, 5 μg. estradiol-cyclopentylpropionate[3] on the 35th day and 35 μg. of relaxin[4] in 0.2 ml. of 1.0% benzopurpurine 4-B[5] on the 43rd day. Twenty-four hours after the administration of relaxin all animals were killed by cervical dislocation and the interpubic ligaments were measured directly with vernier calipers.

## RESULTS

### *Pregnant Mice*

Interpubic ligaments in control mice, measured on the 19th day of gestation, averaged 4.10 mm. (Table 1). Thiouracil markedly suppressed elongation of the interpubic ligament in direct proportion to the dose. Similarly, the response of the symphysis pubis to endogenous relaxin was

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[2] Thyroid powder, U.S.P. Item #196, Wilson Laboratories, Chicago, Illinois.

[3] Depo-estradiol, Upjohn, Kalamazoo, Michigan.

[4] Releasin preparation #80516, Lot M43, each mg. equivalent in activity to 0.12 mg. relaxin Reference Standard; generously supplied by Warner-Chilcott Laboratories, Morris Plains, N. J.

[5] Kindly supplied by Drs. Kroc and Steinetz, Warner-Chilcott Laboratories.

TABLE 1. INFLUENCE OF DIETARY THIOURACIL AND THYROID POWDER SUPPLEMENTS UPON LITTER SIZE, BIRTH WEIGHT AND INTERPUBIC LIGAMENT FORMATION IN 19-DAY PREGNANT MICE

| Group No. | Dietary treatment | No. mice | Fetuses | | Interpubic ligament, mm. |
|---|---|---|---|---|---|
| | | | No. per mother | Ave. birth weight, gm. | |
| I | Fox chow | 15 | 8.6 | 1.05 | 4.10±0.36* |
| II | Fox chow+0.5% thiouracil† | 15 | 8.2 | 1.01 | 2.46±0.22 |
| III | Fox chow+1.0% thiouracil† | 15 | 8.5 | 1.03 | 1.90±0.43 |
| IV | Fox chow+0.2% thyroid† | 15 | 8.0 | 0.94 | 2.32±0.19 |

* Standard error of the mean.
† Supplements added to diet on the 12th day of gestation.

reduced when 0.2% thyroid powder was added to the diet. Neither the average number per litter nor birthweight of the fetuses was significantly affected by either of the drug supplements.

*Virgin Mice*

*Intact:* An average interpubic ligament length of 1.74 mm. (Table 2) was routinely observed in estrogen-primed control mice given 35 μg. of

TABLE 2. INFLUENCE OF DIETARY THIOURACIL AND/OR THYROID POWDER UPON INTERPUBIC LIGAMENT FORMATION IN ESTROGEN-PRIMED, RELAXIN-TREATED MICE†

| Group No. | Operation | Supplement to fox chow diet | No. mice | Interpubic ligament, mm. |
|---|---|---|---|---|
| V | None | None | 37 | 1.74±0.08* |
| | ½ Ovariex | None | 31 | 1.15±0.10 |
| | Ovariex | None | 29 | 1.99±0.14 |
| VI | None | 0.5% thiouracil | 33 | 0.99±0.12 |
| | ½ Ovariex | 0.5% thiouracil | 19 | 0.81±0.14 |
| | Ovariex | 0.5% thiouracil | 25 | 0.52±0.15 |
| VII | None | 1.0% thiouracil | 28 | 0.55±0.15 |
| | ½ Ovariex | 1.0% thiouracil | 21 | 0.28±0.06 |
| | Ovariex | 1.0% thiouracil | 28 | 0.13±0.07 |
| VIII | None | 0.2% thyroid powder | 37 | 2.32±0.13 |
| | ½ Ovariex | 0.2% thyroid powder | 29 | 1.50±0.09 |
| | Ovariex | 0.2% thyroid powder | 28 | 2.17±0.11 |
| IX | None | 1.0% thyroid powder | 27 | 2.12±0.06 |
| | ½ Ovariex | 1.0% thyroid powder | 25 | 1.61±0.09 |
| | Ovariex | 1.0% thyroid powder | 22 | 2.00±0.12 |
| X | None | 0.2% thyroid+0.5% thiouracil | 24 | 1.94±0.07 |
| | ½ Ovariex | 0.2% thyroid+0.5% thiouracil | 21 | 1.36±0.06 |
| | Ovariex | 0.2% thyroid+0.5% thiouracil | 21 | 1.89±0.09 |
| XI | ½ Thyroidx | None | 16 | 0.92±0.06 |
| XII | Thyroidx | None | 15 | 0.00± |

† All mice received 5 μg. estradiol cyclopentylpropionate on day 35, 35 μg. relaxin on day 43, and were killed on day 44.
* Standard error of the mean.

relaxin. As in the pregnant mice, 0.5% thiouracil consistently impaired elongation of the interpubic ligament. Increasing the level of thiouracil administration to 1.0% even further suppressed the average separation of the pubic bones. On the contrary, the response to relaxin administration was significantly increased (2.32 mm., $P < 0.01$) by the addition of 0.2% thyroid powder to the basic diet. When the dietary intake of thyroid powder was further increased to 1.0% there was no additional enhancement, but rather a slight depression of the mean pubic separation seemed to have occurred.

Exogenous relaxin given to mice fed a combination of 0.2% thyroid powder and 0.5% thiouracil in fox chow produced an interpubic separation nearly equal to that recorded for control mice maintained on chow alone.

*Unilateral Ovariectomy:* The removal of one ovary from the system appeared to reduce mean interpubic separations in every instance, regardless of the dietary regimen (Table 2). Only in those mice fed 0.5% thiouracil was this reduction insignificant.

*Bilateral Ovariectomy:* Elongation of the interpubic ligament was somewhat greater following complete ovariectomy in mice maintained on fox chow (1.99 mm. vs. 1.74 mm., $P < 0.02$).

Ovariectomized mice fed either 0.2% or 1.0% thyroid powder and those fed 0.2% thyroid plus 0.5% thiouracil did not appreciably differ, in pelvic responses to relaxin, from their respective intact counterparts. On the other hand, ovariectomy in thiouracil-fed animals markedly depressed the elongation of the interpubic ligament. In fact, this depression was greater than that observed in hemi-ovariectomized mice fed thiouracil.

Except for those mice fed thiouracil, ovariectomy appeared to nearly equalize the response of the symphysis pubis in controls (1.99 mm.), those fed 0.2% or 1.0% thyroid powder (2.17 mm. and 2.0 mm., respectively), as well as those fed thiouracil and thyroid (1.89 mm.).

*Thyroidectomy:* The elongation of interpubic ligaments in mice following *unilateral* thyroidectomy was inhibited to a degree identical to that observed in intact mice fed 0.5% thiouracil. The pubic symphyses of those mice *bilaterally* thyroidectomized remained completely immobile.

## DISCUSSION

The results of this study in pregnant mice indicate that feeding thiouracil or thyroid powder during the last trimester of pregnancy markedly decreases the degree of normally occurring elongation of the interpubic ligament. The absence of effect upon average fetal birthweight is most likely accounted for by the short duration of drug administration.

Likewise, pubic responsiveness to exogenous relaxin is markedly reduced in virgin female mice fed thiouracil. That this inhibition is prob-

bly an effect of sub-normal thyroid activity rather than of the drug *per e* is suggested by the similarity of results in hemi- and bilaterally thyroidectomized mice (Groups XI and XII) compared, respectively, with those n intact mice fed 0.5% and 1.0% thiouracil (Groups VI and VII). In ddition, the diet containing both 0.2% thyroid powder and 0.5% thiourail, which appeared to maintain the animals in an essentially euthyroid ondition, demonstrated no inhibitory effect upon interpubic ligament longation. This finding tends to further deny a direct drug effect of thiuracil.

Although 0.2% thyroid powder partially inhibits pelvic relaxation in 9-day pregnant mice, a diametrically opposite effect appears to occur in he virgin mouse. A possible explanation for this dual influence might be hat the pregnant mouse, already near the upper limit of euthyroid activity, possibly is rendered hyperthyroid by the administration of exogenous thyroid. However, the young nulliparous mouse appears to be secreting thyroid hormone at a sub-maximal rate as evidenced by an accelerated growth rate response to small doses of thyroid (6). Elongation of the interpubic ligament might, therefore, also be augmented by small doses of thyroid at the 0.2% level. In fact, even at the 1% dose level the mice in this study had body weights and interpubic ligament lengths greater than those of controls although essentially equal to those of mice fed 0.2% thyroid. Thyroid powder (0.2%) is capable, nevertheless, of reducing the responsiveness to relaxin in the nulliparous mouse when the period of drug administration is extended from 44 to 88 days (7). The factor of age upon responsiveness must be considered, however, in the interpretation of these latter data.

The decreased degree of interpubic separation of nulliparous mice following hemi-ovariectomy (present study) is not in agreement with the data obtained by Steinetz *et al.* (2) in the 19-day pregnant mouse. These authors felt that the increased extent of separation could be explained by a "decrease in circulating endogenous progesterone with no appreciable decrease in the activity of a relaxative substance" following the removal of one ovary. It seems possible at least that this discrepancy might be accounted for in two ways. First, the differences in time interval between hemi-ovariectomy and sacrifice (4 days, Steinetz (2) as compared to 54 days in the present study) might have permitted changes to occur in the residual ovary which influence levels of circulating progesterone, hormone utilization and target organ sensitivity. Secondly, the obviously different physiological states of the virgin versus the pregnant mouse must be taken into consideration.

The slightly increased response to relaxin in bilaterally ovariectomized *control* mice in the present experiment is consonant with the data of Steinetz *et al.* (2) in similarly operated pregnant mice maintained on progesterone and given exogenous relaxin. Present results also confirm their data

showing that progesterone does, under some circumstances, impair interpubic separation. However, in view of the excellent responses to relaxin in nearly all instances of complete ovariectomy (Groups V, VII, IX and X) there is no support for their thesis that symphyseal separation is dependent upon a delicate balance of relaxin and progesterone.

In consideration of the usual increase in interpubic separation following ovariectomy, it is notable that decreased relaxation occurred in such mice fed thiouracil. This seems clear evidence that this impaired growth of the interpubic ligament is not mediated by the ovary.

Since the action of thiouracil upon symphyseal separation is neither mediated via the ovary nor a direct drug effect, it appears that the thyroid gland may directly influence the structural changes of symphyseal tissue necessary for the elongation of the interpubic ligament. However, that the influences of the thyroid gland upon interpubic separation may be merely reflections of the general physical condition of the animal, is a parameter that must be considered.

*Acknowledgments*

Sincere gratitude is expressed to Drs. R. L. Kroc and B. G. Steinetz, Warner-Chilcott Laboratories, for their generous supplies of Releasin and benzopurpurine and for their continued interest, encouragement and advice in this investigation.

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# IMMUNOLOGICAL INVESTIGATIONS ON BOVINE PITUITARY GROWTH HORMONE

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ABSTRACT

The antigenicity of highly purified bovine pituitary growth hormone was studied by means of anaphylactic shock experiments in guinea pigs, precipitin ring tests with rabbit antisera, and bioassay of the antiserum in hypophysectomized rats for antihormone activity. Bovine growth hormone in the adjuvant, Bayol-Arlacel, was found to be antigenic in guinea pigs and rabbits. Furthermore, as little as 1 μg. of growth hormone could be detected by the precipitin test, using rabbit antiserum which was absorbed free of certain non-specific antibodies. This antiserum did not react with any of the other anterior lobe hormones tested at comparable and considerably higher doses. The absorbed antiserum was found to be capable of inhibiting the stimulation of tibial epiphyseal cartilage and body weight gain effected in hypophysectomized rats by growth hormone.

THE antigenicity of highly purified pituitary growth hormone (somatotropin) has not yet been definitely established. Elberg and Li (1), utilizing a bovine growth hormone preparation that satisfied all the usual criteria for protein purity, concluded, on the basis of precipitin tests with rabbit antiserum and anaphylactic shock experiments in guinea pigs, that their preparation was either non-antigenic or only weakly antigenic, even in the form of the alum precipitate. Morrison *et al.* (2) reported that their electrophoretically homogenous bovine growth hormone, prepared according to the method of Wilhelmi, was antigenic in rabbits. However, the antibody levels represented by their serum dilution titers were so low (1:2 to 1:4) that they are of doubtful significance because of the possible role of contaminants at such low titers.

Ferguson and Boyden (3) demonstrated relatively high hemagglutinating antibody titers against bovine growth hormone when the hormone was administered to sheep over a prolonged period of time. The highest antibody titers were obtained when the animals had received a series of injections with crude anterior pituitary preparations prior to immunization with the purified preparations. On the basis of the findings of these authors, one still cannot exclude the possibility that antibodies not specific for growth hormone could have accounted for a good part of this antibody response. Although they had also employed a growth hormone prep-

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aration which they considered to be pure, no information was presented with respect to its physico-chemical properties nor was any bioassay data reported that would preclude the possibility of contamination with any of the other known anterior pituitary hormones.

In all of the foregoing studies, no attempts were made to correlate the amount of serum antibodies with the degree of inhibition of the biological activity of pituitary growth hormone. In the present investigation, an attempt has been made fully to characterize the hormone preparation employed, and to establish through use of precipitin tests and antihormone bioassay the specificity of the antigen-antibody reaction. The basis for the development of an immunological method of assay for pituitary growth hormone is also presented.

## MATERIALS AND METHODS

### *Hormones*

Highly purified growth hormone (somatotropin, STH) was prepared from ox pituitary glands according to the method of Li (4). The complete characterization of this hormone has been reported elsewhere (5). Other highly purified pituitary hormones utilized to test the specificity of the growth hormone antigen-antibody reaction were ovine growth hormone (6), bovine and sheep lactogenic hormone (prolactin or mammotrophic hormone, MH) (7), bovine thyrotropic hormone (8), ovine (9) and bovine (10) adrenocorticotropic hormone, ovine follicle-stimulating hormone (11), and ovine (12) and bovine (13) interstitial cell-stimulating hormone.

### *Animals*

Young albino female and male rabbits weighing initially approximately 2.4–2.8 kg were employed for the production of antisera. The animals were maintained on rabbit pellets[1] supplemented by fresh greens and carrots at least once a week.

Virgin female guinea pigs weighing initially approximately 375–450 gm. were employed in the experiments dealing with anaphylactic shock. They were maintained on the same pellets that were given to the rabbits but with the addition of rolled barley, supplemented with fresh greens at least once a week.

*Preparation of Antigen for Injection.* In none of the investigations mentioned above has the potent adjuvant Bayol-Arlacel (14), which has been coming into rather wide usage in immunological studies over the last few years, been utilized. In the present investigation, this adjuvant was utilized in all of the experiments dealing with the sensitization of guinea pigs for anaphylactic shock and the immunization of rabbits for the production of antisera. The antigen suspended in adjuvant was prepared as follows: The hormone preparation was ground down to a very fine powder of uniform particles. The required amount was then weighed and suspended in light paraffin oil (Bayol-F) in a small injection vial. After the formation of a homogenous suspension, the desired amount of Arlacel A[2] and Bayol-F was added slowly and the mixture was shaken in the stoppered

[1] Manufactured by California Grain and Milling Company, Oakland, California. Ingredients are Alfalfa meal, ground barley, ground yellow corn meal, wheat middlings, wheat bran, ground oats, soya bean meal, coconut milk, linseed meal, steamed bone meal, salt ½%. Crude protein not less than 16%.

[2] Arlacel A (mannide monooleate) obtained through the courtesy of Atlas Powder Co., Wilmington, Delaware.

vial by hand until a uniform suspension was again obtained. The final proportion of Bayol to Arlacel was usually two parts of the former to three parts of the latter by volume. The antigen was suspended directly in the adjuvant without any aqueous phase, representing a departure from the method given by Cohn (14). In the anaphylactic shock experiments, the antigen in adjuvant was stored under refrigeration over the 3-day period between the two sensitizing injections. For the immunization of the rabbits, the suspension was made up freshly every week.

*Study of Antigenicity of Growth Hormone Preparations.* Three general methods were employed for the study of the antigenicity of the growth hormone preparations: (1) anaphylactic shock experiments, (2) precipitin ring tests, and (3) antihormone bioassay.

For the anaphylactic shock experiments, guinea pigs were sensitized with varying doses of the growth hormone preparation by subcutaneous injection of the hormone in 0.4 cc. of the adjuvant. The sensitizing dose was always repeated 3 days after the initial dose. The animals were then challenged 18–21 days after the first injection, by intracardial injection of the same hormone in 0.5 cc. saline. In order to increase the specificity of the reaction between an antigen and its respective antibody an effort was made to establish (a) the minimal sensitizing dose and (b) the minimal challenge dose required to elicit typical reactions that would lead to fatal anaphylactic shock within 3–5 minutes in the majority of animals tested. It was felt that by determining such minimal dose levels the influence of non-specific trace antigens would be minimized, not only during the sensitization of the guinea pigs, but also during their challenge.

For the precipitin ring tests, rabbits were initially injected subcutaneously with 10 mg. of growth hormone in saline. This was followed by three weekly injections of 10 mg. of hormone suspended in 1 cc. of the adjuvant. The injections were interrupted for from 3 to 4 weeks, and a second series of injections (10 mg. per week) was given for another 3 weeks. After another interval of 3 weeks, a booster injection of 10 mg. was administered, bringing the total amount of hormone injected per rabbit to 80 mg. All animals were bled three weeks later by cardiac puncture. Antibody titers were maintained by booster injections of 5 to 10 mg. of hormone in adjuvant every 4 to 6 weeks. Additional bleedings were made at 2–3 month intervals. Antisera were frozen and stored at −20° C or were preserved with 1 to 10,000 final concentration of merthiolate and stored under refrigeration.

In the precipitin ring tests, the doubling dilution technique was employed in successive tubes. The required amount of antigen was dissolved in saline (.85%) and the pH was adjusted to approximately 7.4 to 7.7 with the addition of 0.1 N sodium hydroxide The first tube of each series usually contained 1.0 mg. or 0.5 mg. of the antigen. Each successive tube contained one-half the amount of antigen contained in the previous tube. The amount of saline in all tubes was 0.5 cc. The highest dilution usually contained 0.5 $\mu$g. antigen. One tenth cc. of antiserum was carefully layered under the antigen solution in each tube by means of 20 gauge spinal needles. The tests were incubated at elevated room temperature (30° C) rather than being placed in the standard 37° water bath, since it was found that the former procedure resulted in less disturbance of the antigen-serum interface. The tests were read after 30 minutes and again after 1 hour of incubation. It was observed that the tube with the highest dilution of antigen giving a positive precipitin ring test would usually shift in the direction of higher dilution by 1 or 2 tubes between these two time intervals. After one hour, further shifts were only infrequently observed.

The standard 4-day assay procedure for growth hormone activity, based upon the tibia test in hypophysectomized rats (15) was employed in the antihormone studies. The ability of the antiserum to inhibit the effect of growth hormone in enhancing the width of the tibial epiphyseal cartilage, constitutes the basis of this assay.

## EXPERIMENTAL AND RESULTS

*Anaphylactic Shock Experiments.* Guinea pigs were injected with highly purified bovine growth hormone (STH) in adjuvant, with doses ranging from 1.0 mg. down to .001 mg. per animal (Table 1). Three weeks later the animals were challenged intracardially with various dose levels of the same hormone. An attempt was thus made to determine the approximate minimal sensitizing dose, as well as the minimal challenge dose required for eliciting the anaphylactic shock response. For the latter, that particular dosage at which the majority of animals showed typical signs of severe anaphylactic shock and died within 3 to 5 minutes following the challenge dose was arbitrarily chosen.

It may be seen from Table 1 that sensitization with 1.0 mg. or .100 mg. of bovine growth hormone resulted in the death of the majority of animals when they were challenged with .100 mg. to .200 mg. of the hormone, whereas a challenge dose of .050 mg. or .025 mg. was apparently insufficient to elicit this response. It is further evident that the sensitizing dose of .001 mg. was too low to induce adequate antibody formation in the guinea pigs, since even relatively high challenge doses (1 to 10 mg.) did not prove fatal to any of the six animals tested. The majority of those sensitized with .010 mg., however, died with typical symptoms when challenged with a 1.0 mg. dose, although a challenge dose of .100 mg. appeared to be insufficient. The results thus suggested that, of the doses tested, the minimal effective sensitizing dose of STH was .010 mg. when the challenge

TABLE 1. DETERMINATION OF THE MINIMAL DOSE FOR SENSITIZATION OF GUINEA PIGS WITH BOVINE PITUITARY GROWTH HORMONE (STH)

| Sensitization with STH | Challenge with STH | |
|---|---|---|
| | Dose | No. dead/No. in gp.* |
| mg. | mg. | |
| — | 2.000 | 0/4 |
| 1.000 | .200 | 4/5 |
| | .100 | 4/6 |
| | .050 | 0/3 |
| | .025 | 0/3 |
| 0.100 | .100 | 3/4 |
| | .050 | 1/4 |
| | .025 | 0/3 |
| 0.010 | 1.000 | 3/4 |
| | 0.100 | 1/4 |
| 0.001 | 1.000 | 0/4 |
| | 5.000 | 0/1 |
| | 10.000 | 0/1 |

* Only typical, severe shock reactions followed by death within 3–5 minutes after challenge were considered here.

dose was 1.0 mg., but with a higher sensitizing dose (.100 mg. or 1.0 mg.), only .100 mg. was required for the minimal effective challenge dose.[3]

*Precipitin Ring Tests.* Both absorbed and unabsorbed growth hormone antisera were employed in the precipitin tests. The antisera from several rabbits were pooled and divided into two portions, one of which was absorbed with lactogenic hormone and normal bovine serum in order to remove certain non-specific antibodies whose presence was suspected on the basis of the anaphylactic shock experiments. Lactogenic hormone was added to the antiserum periodically in very small amounts with aid of micropipettes. After each addition, adequate incubation in the water bath at 37° C was allowed to permit maximal precipitation of antibodies. After each incubation, the precipitating antibodies to the contaminant were removed by centrifugation of the serum. Care was taken not to add any more of the hormone contaminant than was necessary to bring down all the precipitating antibodies to it. This procedure was continued until no further precipitation occurred when small amounts of the hormone were added to the decanted supernatant. A slight excess of the antigen was then added to the resulting absorbed antiserum. The same general procedure was employed with normal bovine serum (1 to 20 dilution) in order to remove antibodies to serum protein(s). The unabsorbed and absorbed antisera were then submitted to ring tests and their reactions with highly purified preparations of various other anterior pituitary hormones were tested. The two principal objectives of the precipitin ring tests were to determine the minimal amount of STH that could be detected with the antisera, and to establish as far as possible the specificity of the STH antigen-antibody reaction.

It can be seen from Table 2 that the unabsorbed growth hormone antiserum gave a positive ring test in the presence of only 1 μg. of the bovine growth hormone. When the antiserum was tested against bovine lactogenic hormone, the highest detectable dilution of this antigen was 2 μg., which suggested that a relatively high antibody response to lactogenic hormone resulted from the immunization of rabbits with the STH preparation. Normal rabbit serum did not give any positive reactions with STH or MH. After absorption of the antiserum with MH and normal bovine serum, it was observed that the test for MH which was previously positive at 2 μg. was now entirely negative, even at the 1.0 mg. concentra-

[3] Animals sensitized to .100 mg. of STH succumbed when challenged with .200 mg. of bovine lactogenic hormone. Preliminary studies had shown us that lactogenic hormone was highly antigenic in rabbits and guinea pigs, confirming earlier reports (16, 17). The minimal effective sensitizing dose was found to be between .0003 mg. and .001 mg. Thus, in this instance, the .100 mg. sensitizing dose of STH must have contained this amount of a substance immunologically indistinguishable from lactogenic hormone, which would suggest a minimal contamination of STH with .3 to 1% of this highly antigenic substance.

TABLE 2. PRECIPITIN RING TESTS WITH BOVINE GROWTH HORMONE ANTISERUM

| Antiserum | Antigen* | Antigen dilution (mg.) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1.000 | .500 | .250 | .125 | .063 | .032 | .016 | .008 | .004 | .002 | .001 | .0005 |
| Not absorbed | STH† | + | + | + | + | + | + | + | + | + | + | + | 0 |
| | MH | + | + | + | + | + | + | + | + | + | + | 0 | 0 |
| | GG | + | + | + | + | + | + | + | + | + | + | + | + |
| | SA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Absorbed‡ | STH | + | + | + | + | + | + | + | + | + | + | + | 0 |
| | MH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | TSH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ACTH | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ICSH | ± | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | FSH | + | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Normal rabbit serum control | STH | 0 | 0 | 0 | 0 | 0 | | | | | | | |
| | MH | 0 | 0 | 0 | 0 | 0 | | | | | | | |
| | GG | 0 | 0 | 0 | 0 | 0 | | | | | | | |

* All antigens are of bovine origin except FSH (sheep). All tests with ovine hormones were negative with the exception of ovine STH, which cross-reacted to almost the same extent as the bovine STH.

† STH = somatotropin (growth hormone); MH = mammotropic hormone (lactogenic hormone, prolactin); GG = gamma globulin; SA = serum albumin; TSH = thyroid stimulating hormone; ACTH = adrenocorticotropic hormone; ICSH = interstitial cell stimulating hormone; FSH = follicle stimulating hormone.

‡ Antiserum was absorbed free of lactogenic hormone antibodies and antibodies to normal bovine serum prior to use in tests.

tion level, whereas STH was still detectable at 1 μg. This indicated that the antibodies to STH were independent of antibodies to MH and normal serum protein(s).

In order to determine whether either bovine gamma globulin (GG) or serum albumin (SA) or both could be the contaminating antigenic serum component(s) in the growth hormone, highly purified preparations of these substances[4] were tested by the ring test against the unabsorbed and the absorbed STH antisera. The unabsorbed antiserum gave a positive test to as low as 0.5 μg. of GG, whereas it gave an entirely negative test to SA. On the other hand, the STH antiserum that had been absorbed with normal bovine serum showed a complete absence of precipitating antibodies to bovine GG. It was thus indicated that antibodies to GG had formed as a result of immunization of rabbits with STH, which in turn suggests the presence of a substance at least similar antigenically to bovine GG in the original STH preparation. Likewise, the detection of antibodies to MH noted above also indicates the presence of a substance in the STH preparation that is immunologically indistinguishable from MH.

Growth hormone antiserum, after being absorbed free of antibodies to the above substances, was then tested against highly purified preparations of other anterior pituitary hormones to determine the specificity of the STH antigen-antibody reaction. The hormones tested were bovine thyroid stimulating hormone (TSH), bovine and ovine adrenocorticotropic hormone (ACTH), interstitial cell stimulating hormone (ICSH), and ovine follicle stimulating hormone (FSH). It may be seen from Table 2 that the antiserum gave a positive test with STH to as low as 1 μg., but tests with other pituitary hormones were negative at comparable or con-

[4] The bovine gamma globulin and serum albumin were obtained through the courtesy of Dr. J. B. Lesh of the Armour Laboratories, Chicago, Illinois.

siderably higher concentrations, with the exception of some weak or doubtful reactions with ICSH and FSH, which took place only at relatively high concentrations (1.0 and 0.5 mg. respectively) of these hormones.

The specificity of the growth hormone antigen-antibody reaction is strongly suggested on the basis of the above precipitin ring tests; the reaction obtained when the antiserum is tested with STH does not represent a reaction of the antiserum to any of the other anterior lobe hormones tested, and thus the reaction with the absorbed antiserum which detects 1 μg. of STH can probably be taken to be specific for STH.

In an attempt to make a practical application of the above findings, the absorbed STH antiserum was employed in precipitin tests against highly purified bovine TSH (8) to determine the STH content, if any, in the latter hormone. It was observed that this antiserum, which could detect 1 μg. of STH, did not give a positive test when layered under 2.0 mg. of TSH in 0.5 cc. of saline. This suggested that 2.0 mg. of bovine TSH did not contain as much as 1 μg. of STH, an amount which would be equivalent to a contamination of less than five one-hundredths of 1%. In order to determine whether or not the preponderance of TSH molecules could mask or interfere with the detection of such a minute amount of STH, increasing amounts of STH (1, 2 and 4 μg.) were added to successive tubes containing 1.0 mg. each of bovine TSH in 0.5 cc. of saline. The STH antiserum was then layered under the antigen phase and the tests were read after the usual incubation period. All tests were positive, including the tube containing only 1 μg. of STH, while similar tests set up with normal rabbit serum were completely negative. This experiment had thus demonstrated that if as little as 1 μg. of STH is present in a mixed solution containing a large excess of another hormone (1.0 mg. of TSH), the STH can still be detected with 0.1 cc. of the specific antiserum.

*Bioassay for Antihormone Activity.* Seventy-eight female rats of the Long-Evans strain were hypophysectomized at twenty-eight days of age. Two weeks postoperatively the rats were apportioned into 13 uniform groups on the basis of weight, each group containing 6 animals (Table 3). The average initial body weight at this time was approximately 80 gm. per animal in all groups. Group 1 served as saline control. All other groups were injected with either normal rabbit serum or antiserum. Each of the animals in Group 2 received 1 cc. daily of normal rabbit serum only, while those in Group 3 received the same amount of rabbit antiserum only. Group 4 received daily injections of 1 cc. of normal serum to which .012 mg. of STH had been added, and Group 5 received 1 cc. of antiserum plus the same amount of STH. The mixtures were injected intraperitoneally immediately after the addition of hormone to the serum. With Groups 6, 7 and 8, the same daily dose of hormone was added to the 1 cc. of antiserum in the same manner as it was with the two preceding groups but

TABLE 3. ANTIHORMONE ASSAY IN HYPOPHYSECTOMIZED RATS INJECTED WITH STH AND STH ANTISERUM

| Experimental group* | Average initial weight | Average weight change | Tibia width |
|---|---|---|---|
| | gm. | gm. | $\mu \pm$S.E. |
| 1. Saline (i.p.) | 78.0 | −1.3 | 161 ±2.66 |
| 2. Normal serum (i.p.) | 79.7 | −1.3 | 160 ±1.09 |
| 3. Antiserum (i.p.) | 76.0 | −2.0 | 157 ±3.50 |
| 4. STH+NS (immed. injec.) (i.p.) | 79.3 | +4.4 | 242 ±2.45 |
| 5. STH+AS (immed. injec.) (i.p.) | 78.5 | +0.2 | 178 ±6.25 |
| 6. STH+AS (incub. 1 hr.)† (i.p.) | 78.0 | −1.7 | 170 ±8.07 |
| 7. STH+NS (incub. 1 hr.)† (i.p.) | 78.8 | +4.8 | 249 ±3.46 |
| 8. STH+AS (incub. 1 hr.)‡ (i.p.) | 79.7 | −1.2 | 154 ±4.75 |
| 9. STH (s.c.)+AS (undil.) (i.p.) | 82.0 | −1.7 | 152 ±2.34 |
| 10. STH (s.c.)+AS 1:2 (i.p.) | 81.0 | +0.5 | 163 ±3.43 |
| 11. STH (s.c.)+AS 1:4 (i.p.) | 82.5 | +0.2 | 170 ±2.32 |
| 12. STH (s.c.)+AS 1:8 (i.p.) | 78.8 | +0.3 | 168 ±6.52 |
| 13. STH (s.c.)+AS 1:16 (i.p.) | 79.0 | +2.5 | 207 ±2.16 |

* STH = bovine somatotropin (growth hormone); NS = normal rabbit serum; AS = rabbit antiserum; s.c. = subcutaneously; i.p. = intraperitoneally. All experimental groups consisted of 6 rats per group. STH dosage was .012 mg. daily, added to serum, ×4 days (Groups 4–8); and the same dosage in 0.5 cc. saline, given subcutaneously (Groups 9–13). Daily dose of normal serum or antiserum was 1 cc. intraperitoneally, ×5 days, starting on the day before the beginning of hormone injections.

† Incubation for 1 hr. at 37° C.

‡ After incubation, the serum was centrifuged to remove the precipitate, and only the supernatant was injected.

the mixture was incubated at 37° C for 1 hour prior to injection in order to allow a near-maximal degree of precipitation of antigen-antibody complexes. Of these latter 3 groups, Groups 6 and 7 were injected with the entire mixture after shaking to insure a uniform distribution of precipitate particles, and Group 8 was injected with the clear supernatant fluid only, after centrifugation and removal of the precipitate. All injections in Groups 1 through 8 were made intraperitoneally. In the last 5 groups (9 through 13) the hormone and serum were injected each into 2 different sites, the former subcutaneously in the dorsum, and the latter intraperitoneally. These groups received STH in daily doses of .012 mg. in 0.5 cc. saline, and varying dilutions of the antiserum (1 cc.) ranging from the undiluted material to a 1:16 dilution. STH was injected daily for 4 days. Serum injections were started on the day before the hormone injections were begun, and were administered in 5 daily injections. All animals were sacrificed approximately 20–24 hours after the final injection.

It may be seen from Table 3 that the daily dose of .012 mg. of STH plus normal serum resulted in a highly significant increase ($P < .001$) in the tibial epiphyseal plate width (242 micra) over that of the hypophysectomized controls (160 micra). The injection of normal serum or antiserum alone had no significant effect upon the tibia plate width. The results in Groups 5 through 8 indicated that the antiserum was capable of neutralizing completely the growth hormone activity, and that the 1-hour period of incubation was not necessary for this neutralization. It was observed

that the incubation of STH with normal serum for 1 hour at 37° C did not result in any decrease in hormone activity. It is clear that 1 cc. of the various antiserum dilutions, up to and including the 1:8 dilution, exerted a significant ($P < .001$) antihormone effect sufficient to completely neutralize the enhancement of tibial growth produced by the .012 mg. daily dose of STH. With the 1:16 dilution, there was still significant ($P < .001$) although incomplete neutralization (207 micra) of the growth

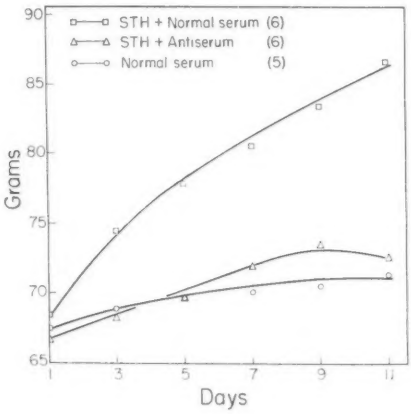


FIG. 1. The influence of STH antiserum on body weight gain of STH-treated hypophysectomized rats. Animals were injected daily with 0.1 mg. STH in 0.5 cc. saline (s.c.) and 1 cc. serum (i.p.) ×10 days. Numbers in parentheses indicate number of rats per group.

hormone effect. It was noted that the changes in tibial cartilage width were generally reflected in body weight changes, even in this short-term assay.

The influence of the antiserum on body weight gain was well demonstrated in a 10-day assay[5] in which animals similar to those used in the above 4-day test were employed. Injections were begun 2 weeks postoperatively. The STH dosage was increased from .012 mg. to .100 mg. daily. The antiserum dosage was maintained at 1 cc. daily, since the results of the 4-day test had indicated that this amount of undiluted antiserum should be just sufficient to neutralize a daily dosage of .100 mg. STH. The hormone was injected subcutaneously in the dorsum while the serum was administered intraperitoneally. The inhibitory effect exercised by the antiserum on the body weight gain of STH-injected rats is shown in Figure 1. The STH group gained an average of 18 grams per rat over the 10-day period, while in the group that received the antiserum in addition to the hormone there was a marked if not complete inhibition of the STH stimulation. With respect to tibial growth, the results of the 10-day assay were almost identical to those obtained in the 4-day assay.

[5] The authors are grateful to Robert N. Cathcart and Carl D. Shirk for providing us with the data for the 10-day assay. A detailed report including a histological study will be presented in a subsequent publication.

## DISCUSSION

The results of this investigation have clearly demonstrated the antigenicity of the bovine pituitary growth hormone preparation in guinea pigs and rabbits when the adjuvant, Bayol-Arlacel, was employed as a vehicle. The experiments which were concerned with the anaphylactic shock response in guinea pigs merely indicated that antibodies had formed to some substance(s) in the hormone preparation. However, the precipitin ring tests revealed that at least three different sets of antibodies were formed when rabbits were immunized with the highly purified bovine growth hormone preparation; namely, antibodies against bovine lactogenic hormone, against some normal bovine serum component(s), and presumably against the growth hormone itself. Although the detection of antibodies to the substances other than growth hormone was unexpected, it is understandable, since it is known that lactogenic hormone and serum proteins are highly effective antigens when injected into the proper species. The trace amounts of these substances present were magnified disproportionately by the immunization methods employed, particularly because of their relatively high antigenicity in comparison with the growth hormone itself. However, without having used such techniques it might not have been possible to obtain an antiserum with such marked antihormone properties.

It was of great interest to observe that the growth hormone antiserum when absorbed free of antibodies to the above non-specific substances could still detect the presence of 1 $\mu$g. of growth hormone by the precipitin test whereas it did not react with any of the other anterior lobe hormones at comparable and much higher doses. The fact that this same absorbed antiserum possessed a marked capacity to neutralize growth hormone activity furnishes strong supporting evidence for the specificity of the antigen-antibody reaction in question.

The possibility of practical application of the above findings to the assay of hormones in tissues and biological fluids is now being investigated. Studies are also in progress to determine similarities and differences among the pituitary growth hormones from several species including those of man, monkey, whale, sheep, pig and ox.

In addition, we have observed that human pituitary growth hormone is also antigenic in rabbits and guinea pigs. A complete report on this aspect will be presented elsewhere.

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# NOTES AND COMMENTS

## THE EFFECTS OF ESTRONE ON THE RESPONSE TO TESTOSTERONE PROPIONATE IN CASTRATED MALE RATS


### ABSTRACT

Data are presented on the responses to a series of combinations of estrone and testosterone propionate over a wide range of doses. Both body weight gain and levator ani weights reflect the catabolic character of the response to estrogens and the anabolic effects of androgens. The seminal vesicles respond to both androgens and estrogens, but the response to testosterone propionate far exceeded the response to estrone. The ventral prostate, on the other hand, responded only to the androgen. The effects of several other estrogenic and non-estrogenic steroids are also discussed.


Estrogens as well as androgens have stimulatory effects on the secondary sex glands of castrated male rats. Histologically, the responses can be shown to differ. Some of these effects were briefly reviewed by Dorfman and Shipley (1). Estrogens administered along with androgens produced variable effects (2) .We have studied the effects of combinations of estrone and testosterone propionate over a wide range of doses, using 4 endpoints for comparison.

Albino rats of the Sprague-Dawley strain were castrated at 23 days of age. Starting 18 days later, they were injected intramuscularly with the steroids in oil over a period of 7 days. They were sacrificed on the day after the last injection and the organs were dissected out and weighed on a torsion balance. The data are presented in Table 1.

### RESULTS

*Body Weight.* Testosterone propionate by itself usually produced an average gain in body weight which was above the control average although, in general, these increases were not significant. On the other hand, treatment with estrone, even at the lowest dose, reduced the gain in body weight as compared to the controls. At any dose of estrone, testosterone propionate tended to counteract in part the catabolic effect of the former compound.

*Seminal Vesicles.* All doses of both estrone and testosterone propionate caused an increase in average seminal vesicle weights. However, the slopes of the dose response curves for the two steroids differed markedly. At each dose level of testosterone propionate, the addition of estrone caused an increase in the seminal vesicle response. At the higher levels of testosterone propionate, this augmenting effect of estrone was independent of the dose of estrone over the range of total doses from 0.05 to 1.0 mg.; at the lower doses of testosterone propionate, the size of the dose of estrone may have influenced the degree of response.

*Ventral Prostate Gland.* While testosterone propionate caused increases in the weights of the ventral prostate glands similar in extent to those seen in the seminal vesicles, estrone had no effect on the weight of this gland. Furthermore, the addition of estrone to testosterone propionate did not influence the response of the ventral prostate to the later steroid.

*Levator Ani Muscle.* Testosterone propionate produced the well-known anabolic effect as measured by the increase in the weight of this muscle. Estrone, on the other hand,

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TABLE 1. EFFECT OF ESTRONE ON THE RESPONSE OF CASTRATE RATS TO TESTOSTERONE PROPIONATE

Each figure is the average value for a group of 8 rats

| Total dose | | Body weight gain | Seminal vesicles | Ventral prostate | Levator ani |
|---|---|---|---|---|---|
| Estrone | Testosterone propionate | | | | |
| mg. | mg. | g. ±S.E. | mg. ±S.E. | mg. ±S.E. | mg. ±S.E. |
| .0 | .0 | 31.4 ±1.7 | 11.8 ± .8 | 14.1 ±1.4 | 74.5 ± 4.3 |
| .0 | .05 | 35.9 ±1.7 | 16.0 ± .5 | 20.6 ± .8 | 83.4 ± 3.4 |
| .0 | .1 | 33.6 ±2.1 | 30.2 ± 1.0 | 26.4 ±1.2 | 79.2 ± 5.4 |
| .0 | .2 | 31.0 ±3.3 | 56.1 ± 2.9 | 54.0 ±2.9 | 101.1 ± 5.9 |
| .0 | .5 | 38.0 ±1.5 | 95.7 ± 5.2 | 106.0 ±4.2 | 139.6 ± 8.4 |
| .0 | 1.0 | 37.3 ±1.5 | 130.1 ± 6.2 | 114.5 ±3.9 | 120.9 ± 5.4 |
| .05 | .0 | 14.8 ±1.4 | 21.0 ± .8 | 13.2 ±1.2 | 61.2 ± 3.1 |
| .05 | .05 | 21.1 ±1.7 | 24.7 ± .8 | 17.8 ±1.5 | 56.0 ± 3.2 |
| .05 | .1 | 27.4 ±6.1 | 38.2 ± 2.0 | 25.9 ±1.5 | 73.9 ± 4.0 |
| .05 | .2 | 24.8 ±1.8 | 70.9 ± 3.5 | 47.8 ± .9 | 91.1 ± 3.3 |
| .05 | .5 | 26.3 ±1.1 | 135.4 ± 4.1 | 92.5 ±4.5 | 115.8 ±16.6 |
| .05 | 1.0 | 29.8 ±1.4 | 178.5 ± 7.2 | 144.0 ±7.6 | 156.7 ± 6.0 |
| .1 | .0 | 18.1 ±1.4 | 22.8 ± 1.8 | 16.5 ± .8 | 77.6 ± 6.5 |
| .1 | .05 | 18.6 ±1.3 | 39.2 ± 2.4 | 28.4 ±2.1 | 81.0 ± 5.1 |
| .1 | .1 | 18.9 ±1.5 | 42.2 ± 2.0 | 27.5 ±1.4 | 65.1 ± 3.7 |
| .1 | .2 | 19.9 ±2.1 | 77.1 ± 5.4 | 44.6 ±3.5 | 85.4 ± 5.8 |
| .1 | .5 | 26.1 ±1.7 | 130.3 5.3 | 89.5 ±2.2 | 132.8 ± 8.3 |
| .1 | 1.0 | 25.6 ±4.0 | 174.3 ± 8.8 | 142.5 ±6.7 | 147.6 ± 3.8 |
| .2 | .0 | 14.4 ±1.3 | 25.7 ± .8 | 15.0 ± .7 | 60.8 ± 2.2 |
| .2 | .05 | 18.8 ±1.3 | 35.6 ± 1.2 | 22.0 ±1.5 | 72.7 ± 3.1 |
| .2 | .1 | 10.5 ±1.9 | 43.1 ± .7 | 28.2 ±1.8 | 55.1 ± 3.5 |
| .2 | .2 | 15.9 ±1.3 | 86.7 ± 2.7 | 59.8 ±2.3 | 99.8 ± 5.6 |
| .2 | .5 | 20.8 ±1.4 | 139.0 ± 3.3 | 103.4 ±4.7 | 128.1 ± 4.2 |
| .2 | 1.0 | 25.8 ±2.4 | 216.3 ±11.0 | 146.5 ±8.7 | 184.0 ±32.2 |
| .5 | .0 | 8.4 ±2.2 | 30.5 ± 1.8 | 18.3 ±1.3 | 65.9 ± 4.5 |
| .5 | .05 | 11.5 ±2.1 | 31.2 ± 1.3 | 17.3 ± .8 | 64.5 ± 2.1 |
| .5 | .1 | 8.9 ±1.4 | 51.3 ± 2.9 | 33.9 ±2.8 | 65.7 ± 2.8 |
| .5 | .2 | 14.6 ±2.6 | 87.8 ± 2.2 | 51.8 ±3.7 | 94.8 ± 2.7 |
| .5 | .5 | 9.8 ±2.3 | 133.6 ± 5.3 | 84.0 ±4.9 | 105.8 ± 8.4 |
| .5 | 1.0 | 22.8 ±1.6 | 180.6 ± 8.5 | 118.8 ±3.8 | 127.7 ± 5.1 |
| 1.0 | .0 | 9.4 ±1.5 | 30.7 ± 1.8 | 14.8 ± .9 | 57.6 ± 3.0 |
| 1.0 | .05 | 8.5 ±2.5 | 32.3 ± .5 | 21.1 ±1.3 | 63.3 ± 4.2 |
| 1.0 | .1 | 5.3 ±2.7 | 59.5 ± 1.7 | 40.1 ±2.9 | 85.2 ± 5.0 |
| 1.0 | .2 | 6.8 ±1.9 | 77.7 ± 2.7 | 52.5 ±2.5 | 64.5 ± 3.1 |
| 1.0 | .5 | 15.1 ±2.1 | 143.7 ± 7.6 | 100.1 ±3.0 | 119.5 ± 6.4 |
| 1.0 | 1.0 | 24.4 ±1.8 | 171.0 ± 4.7 | 106.0 ±4.0 | 139.1 ± 3.3 |

caused a reduction in weight which appears to be a reflection of the general catabolic effect of this steroid. From the table it is evident that the higher doses of testosterone propionate completely counteracted the catabolic effects of estrone on the levator ani muscle.

*Other Steroids.* The effects of a number of other estrogens on the response to a fixed dose of 0.5 mg. testosterone propionate were studied. Diethylstilbestrol, estradiol and Vallestril,[1] in total doses of 0.2 to 2 mg., were catabolic as measured by change in body weight, but augmented the seminal vesicle response. Several non-estrogenic steroids were also studied. Cortisone acetate at a total dose of 5 mg. was catabolic without inducing an increased seminal vesicle response. Progesterone at doses of 0.5 to 10 mg. was not cata-

[1] Vallestril, 3-(6-methoxy-2-naphthyl) 2,2-dimethylpentanoic acid. Searle.

bolic and did not alter the seminal vesicle response. None of these significantly altered the responses of the ventral prostate glands or the levator ani muscles to testosterone propionate.

In another series, 17$\alpha$-ethyl-19-nortestosterone was substituted for testosterone propionate. This compound showed only minimal androgenic effects as measured by increased weights of the seminal vesicles and ventral prostate gland. Significant increases in the weights of the levator ani muscles were observed. Combined with this compound at a total dose of 1 mg., estrone at doses of 0.2 to 2 mg. was increasingly catabolic while at the same time augmenting the seminal vesicle response. Estrone had no effect on the responses of the ventral prostate glands or levator ani muscles to 17$\alpha$-ethyl-19-nortestosterone.

### COMMENT

While the seminal vesicles showed a response to both estrogens and androgens the ventral prostates responded only to the latter. In the male rat estrogens are catabolic as shown by the marked inhibition in the gain in body weight. The androgenic-anabolic steroids tend to overcome this effect and stimulate growth of the levator ani muscle. In the study of new synthetic steroids, valuable information regarding their hormonal activities can be obtained by measuring these four parameters.

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## FAILURE OF SURAMIN (BAYER 205; GERMANIN) TO CAUSE CHANGES IN THE ADRENAL CORTEX OF THE RAT[1,2]

### ABSTRACT

An attempt was made to reproduce the experiments of Humphreys and Donaldson in which histological lesions in the adrenal cortices of rats had been produced by repeated injections of the organic compound suramin (Bayer 205). In spite of a considerable mortality among the injected rats in the present experiment, no adrenal lesions could be demonstrated.

Suramin is a compound synthesized by the I. G. Farben industry in 1905, and long used as a trypanosomacide. Its chemical designation is: hexasodium *sym.*-bis (*m*-amino-benzoyl-*m*-amino-*p*-methyl-benzoyl-1-naphthylamino-4,6,8-trisulfonate) carbamide. In

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[1] The authors are indebted to Dr. Tyree C. Wyatt for assistance in the histological aspects of the study. Thanks are due to Mrs. Helen Phippen and Miss Anita Dates for technical assistance.

[2] Suramin sodium (Naphuride® sodium) was generously supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

the late 1940's, it was tried empirically in the treatment of pemphigus by British and French physicians. Mahoney and Barrie, in 1950, reported on a case of adrenal insufficiency after suramin therapy of pemphigus (1). This was followed by similar papers reporting new cases of adrenal insufficiency in patients after suramin treatment (2–6).

In 1942, Humphreys and Donaldson described the effects of suramin on rats (7). They described in their animals an acute degenerative band $\frac{1}{3}$ to $\frac{1}{4}$ the width of the adrenal cortex, involving the fasciculata and glomerulosa. These workers did not describe the relationship between length of treatment and appearance of lesions, nor were chemical determinations done on the adrenals, or histochemical techniques applied.

The purpose of the present experiment was to make further studies of the effects of suramin on the adrenal cortex of rats. In this experiment white, male Sprague-Dawley rats with a weight range of 90 to 110 gm. were used. They were divided into 2 groups of 18 rats each: the control group, which received injections of 0.3 ml. of sterile saline subcutaneously every other day, and the experimental group, which was given subcutaneously a 2 per cent, aqueous suramin solution in the dose of 4 mg. per 100 gm. body weight every other day. This dosage was determined from the data of Humphreys and Donaldson (7). The animals were maintained on this schedule of injections for periods of time up to 21 days. Groups were sacrificed after 1, 5, 12, 14, and 21 days of injections. When sacrificed, the adrenals were removed by micro-dissection, kept moist and weighed on a Roller-Smith torsion balance. The left adrenal was quick-frozen for chemical determinations and the right adrenal was placed in 10% formalin for histological study.

Of the 18 animals given injections of suramin, 6 of them died spontaneously during the course of injections. Most of the experimental group showed marked weight loss after 5 doses of suramin. On 7 of the experimental group, and on 4 of the control group histological preparations were made of salivary glands, liver, spleen, pancreas, heart, lungs, testes, epididymus and thymus. The tissues were stained with haematoxylin and eosin. No histological differences between the two groups were observed. Adrenals from the experimental and control groups were sectioned on the freezing microtome and examined for sudanophilic and birefringent material. There were no observable differences either in location or total amounts of these materials in the adrenals of the control and experimental animals. In addition no difference between the two groups could be detected with respect to: a) adrenal weights, b) adrenal total cholesterol content, and c) per cent of cholesterol esters in adrenal tissue. The ratio of adrenal weight to total body weight was consistently elevated in the experimental group, secondary to the marked total body weight loss of this group.

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# THE ENDOCRINE SOCIETY

## 1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . . )." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

## 1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

### Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

### The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

### The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

### The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

*Nominations*

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

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## AMERICAN UROLOGICAL ASSOCIATION, INC.

*Urology Award.* The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300 and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition is limited to urologists who have been graduated not more than ten years, and to hospital internes and residents doing research work in Urology.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Chalfonte-Haddon Hall, Atlantic City, New Jersey, April 20–23, 1959.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before December 1, 1958.

## THIRD WORLD CONGRESS ON FERTILITY AND STERILITY, AMSTERDAM, JUNE 7 TO 13, 1959

The themes of the congress are:

1. Embryonal death.
2. Hormonal factors and vitamins in fertility and sterility.
3. Relative value of the techniques for the study of the endocrine functions in human sterility.
4. Biochemistry of spermatogenesis.
5. Psycho-sexological problems in sterility.

The Registration Fee is $40.00 for full members and $15.00 for associate members. Titles for papers should be submitted to Dr. Alfonso Alvarez-Bravo in Mexico City 5, D.F., Mexico, or to Prof. Dr. B. S. ten Berge, c/o Academic Hospital, Groningen, the Netherlands. For further information and registration, prospective participants should apply to the Secretariat of the Third World Congress on Fertility and Sterility, or to the nearest Wagons-Lits/Cook office.